IRON METABOLISM IN RELATION TO NUTRITION

By

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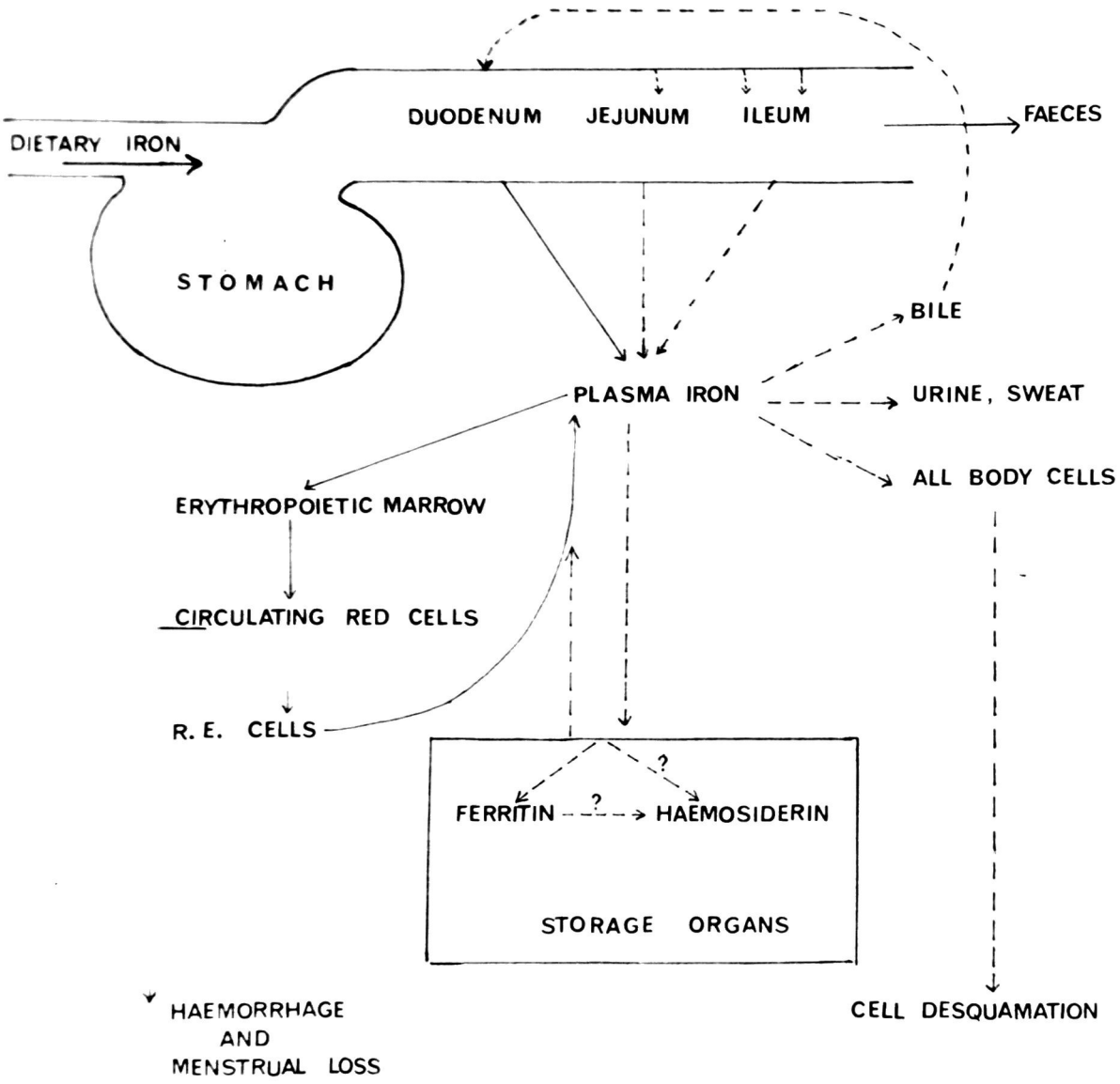
INTRODUCTION

I. IRON METABOLISM

Iron is present in so many compounds of physiological importance (e.g., haemoglobin, myoglobin, ferritin, cytochromes, flavoproteins such as xanthine oxidase) that it is not surprising its metabolism has attracted a great deal of research. The fact that disorders of iron metabolism have clinical importance has provided an additional stimulus. Many of the biologically interesting compounds of iron are proteins, so that it is reasonable to inquire into possible metabolic inter-relationships. This thesis reports a contribution to the study of the effects of protein deficiency on iron metabolism with particular reference to absorption and storage.

FIG. A

MAIN FEATURES OF IRON METABOLISM IN ADULT HUMAN



The total amount of iron in the body of a normal man has been estimated at about 5 g., 60 to 87.5 mg. per kilogram of body weight (Widdowson, McCance and Spray, 1951). Haemoglobin iron constitutes 70-80% of the total (3.5 - 4 g.) and storage iron 20 - 30% about 1 - 1.5 g. (Bogniard and Whipple, 1932; Hahn, 1937; Drabkin, 1951). All the rest plasma, myoglobin, intracellular enzymes - amounts to only a few hundred milligrams.

The main features of iron metabolism in the normal subject, now well established are summarized in Fig. A. Absorption of iron and its loss from the body are both small (1 - 3 mg. per day for an adult human depending on sex) by comparison with the quantity utilized daily for haemoglobin synthesis and liberated in haemoglobin catabolism (25 mg.). Under normal conditions there is almost complete re-utilization of iron for haemoglobin synthesis, and therefore very little exchange with stored iron, (Dubach, Moore, and Minnich, 1946; Finch, Hegsted, Kinney, Thomas, Rath, Haskins, Finch, and Fluharty, 1950; Josephs, 1958; Moore, 1959-60). These facts have been brought out in reviews by Finch et al. (1950) Josephs (1958) and Moore (1959-60).

II. STORAGE IRON

Storage iron may be defined as the iron which can be mobilized from various tissues for haemoglobin synthesis when needed. Under normal conditions it is found to comprise 20 - 30% of the total body iron, about 1 - 1.5 g. (Bogniard and Whipple, 1932; Hahn, 1937; Drabkin, 1951). Such iron is stored intracellularly as ferritin and haemosiderin (Neumann, 1888; Laufberger, 1937; Hahn, Granick, Ball and Michaelis, 1943; Granick, 1951; Shoden, Gabrio and Finch, 1953). Some chemical data show a degree of similarity between these two compounds (Cook, 1929; Granick, 1949; Shoden, Gabrio, and Finch, 1953). Ferritin was first isolated from horse spleen in 1934 by Laufberger, who also crystallized it with cadmium sulphate (1937). It was found to consist of a homogeneous protein, apoferritin, in combination with up to 23% iron (Granick, 1946). Haemosiderin is the name originally given by Neumann (1888) to the iron rich granules found in the spleen.

They could be seen under the microscope and gave a positive Prussian blue test. Little is known of the composition or properties of haemosiderin, although it is usually considered to contain more iron and less nitrogen than ferritin (Behren and Asher, 1933; Asher, 1933; Ludewig, 1957; McKay and Fineberg, 1958; Richter, 1960; Shoden and Sturgeon, 1960). The interrelation of these compounds is not clear, but both increase in the tissues when excess iron is given, and both become depleted when iron required for haemopoiesis is not available in the diet (Finch, Hegsted, Kinney, Thomas, Rath, Haskins, Finch and Fluharty, 1950). It has been suggested that ferritin iron may be more readily available than haemosiderin iron, but evidence for this is incomplete.

Storage iron is widely distributed throughout the body. The main localities are the liver, spleen, bone marrow, other reticulo-endothelial cells, glandular tissues of the pancreas, adrenals and other secretory cells. These sites are named in an order corresponding roughly to their relative quantitative importance.

There are various ways of determining the amount of storage iron in the body. It may be measured by forced depletion of the circulatory haemoglobin (Finch, et al. 1950) and calculation of the extent to which storage iron contributes to replenishment. This procedure can be used in observations on human subjects, but with experimental animals chemical analysis of the individual organs or even entire carcases becomes possible. Errors may result from incorrect allowances for the quantity of haemoglobin present. In special circumstances useful semi-quantitative information may be obtainable from histological examination of human biopsy specimens, usually liver and bone marrow (Rath and Finch, 1948; Davidson and Jennison, 1952; Beutler, Dreman and Block, 1954 and Gillman, Lamont, Hathorn and Canham, 1957).

III. PROTEIN DEFICIENCY AND IRON METABOLISM

Malnutrition in its acute and chronic forms is widely spread among the populations of Africa, Asia, and Central and South America. Owing to their poverty and low economic status, these people cannot afford the expensive, well balanced diets essential for normal growth and development. They are thus obliged to live on cheap, mainly carbohydrate diets consisting, in some cases, solely of the locally grown cereals, usually maize. Due to lack of high quality proteins and sources of important vitamins from the food, multiple dietary deficiency diseases may develop. Kwashiorkor, liver diseases, pancreatic disease, nutritional anaemias, due to protein deficiency, and scurvy, pellagra, Beri-Beri, and pancreatic degeneration, due to vitamin deficiences, are some examples of the nutritional disorders widely spread among these people. Parasitic infestation of the bowel often complicates these conditions.

Deficiencies of vitamins such as pyridoxine, ascorbic acid and nicotinamide are well known to affect iron metabolism in various interesting ways, (Vorloop and Rademaker, 1960; Cartwright, Wintrobe and Humphreys, 1944; Gillman and Gillman, 1944; Harris, Whittington, Weisman, and Horrigan, 1956; Bothwell, Bradlow, Jacobs, Keeley, Kramer, Seftel and Zail, 1964), but discussion of these is beyond the scope of the present work.

In protein deficiency and in malnutrition in general, every organ and cell in the body must be affected but not necessarily all at the same rate or at the same time. There is not as yet a full picture of how each system participates in the total effect, but work on rats (Shils and Stewart, 1954; and Shils, Friedland and Stewart, 1954) and monkeys (Ramalingawami, 1964) has shown that visceral organs appear to contribute more than their fair share to the general loss in weight.

Kwashiorkor is a manifestation of protein deficiency common in children of malnourished communities (Williams, 1935, 1938; Smith, 1942 - 43; Smith, 1952; Meiklejohn and Passmore, 1951; Brock and Autret, 1952; Trowell, Davies and Dean, 1954; Zuidema, 1959; Sharper, 1960; Brock, 1961). In this condition, also, the metabolism of all tissues and cells is disrupted to a lesser or greater degree (Trowell et al. 1954; Brock and Autret, 1952; Brock, 1961). Among the organs attacked by malnutrition, the liver, pancreas and intestinal mucosa are the most seriously affected (Brock, 1961; Ramalingawami, 1964), fatty livers, cirrhotic livers, and siderotic livers have all been reported in protein malnourished people (Gillman, Gillman, Inglis, Friedlander and Hamman, 1944; Gillman and Gillman, 1944; Brock and Autret, 1952; Trowell et al. 1954). Pancreatic lesions were reported by Davies (1948), Veghelyi (1948), Veghelyi, Kemeny, Pozsonyi, and Sos (1950) and pancreatic fibrosis and calcification were observed by Sharper (1960) in East African natives suffering from protein malnutrition. Zuidema (1959) reported several similar cases in Indonesians.

In Kwashiorkor and in protein malnutrition in general it is usual to find some degree of anaemia, the type and severity of which depend primarily on whether a deficiency of iron, vitamin B_{12} or folic acid is also present, or whether parasitic infestation of the bowel is superimposed. Storage iron may also be increased or decreased depending on the previous dietary history of the patient (Scrimshaw and Behar, 1961).

The discovery of the high incidence of haemosiderosis in some South African Bantu tribes (Strachan, 1929; Gillman, Mandelstam and Gillman, 1945) opened a new sphere of research on the relation of iron metabolism and malnutrition. Abnormal deposition of iron in organs and tissues of adult South African Bantu was first reported by Strachan (1929) who studied a number of cases of haemosiderosis with special reference to its aetiology. He reported:-

"..... Haemochromatosis is a not uncommon disease in the South African natives. The chief factor in its production appears to be the diet, in which there is excess of carbohydrate and probable contamination with salts of zinc and of tin ....."

Gillman et al. (1945) reported chemical and histological studies on livers from 500 consecutive post-mortems. They concluded that siderosis with or without cirrhosis, such as seen in Africans in Johannesburg was indistinguishable from the liver lesions described in confirmed cases of haemochromatosis, and that the accumulation of iron in the liver represented only a part of a widespread disturbance in mineral metabolism resulting from malnutrition and not from an inborn error of metabolism. Gillman, Lamont Hathorn and Canham (1957) extended these results with a report on 100 liver biopsies done in Durban on African males suspected to have liver disease, when distinct siderosis was found

in eighty-eight. They came to the conclusion that malnutrition common among natives of South Africa caused widespread disorders of cell metabolism leading to abnormal absorption and deposition of iron in the tissues.

Higginson, Gerritsen and Walker, (1953) and Walker and Arvidsson (1953) held a different view from that of the Gillman's (1945, 1957). From their results of anatomical histological and chemical investigations of siderosis in the Bantu, they concluded that iron deposition occurred principally in the reticulo-endothelial system and not until heavy deposits were present did the element appear in epithelial tissues. In classical haemochromatosis deposition in hepatic parenchyma predominates over deposition in the reticulo-endothelial cells. Walker and Arvidsson (1953) found a very high iron intake by the Bantu, as much as 200 mg. daily due to uptake from iron utensils used for preparation

of their usual food, and especially for fermented cereal products called "Kaffir Beer". These workers and Higginson et al. (1953) suggested the aetiology of this condition to be possibly the oral iron overload. They doubted whether under nutrition or malnutrition could be regarded as a major aetiologic factor, though they did not exclude the possibility.

Much experimental work in this field seems to support the idea that certain kinds of malnutrition may promote the absorption of iron. Hegsted, Finch and Kinney (1949) reported excessive absorption and deposition of iron in the livers of rats fed a protein-deficient diet supplemented with iron when compared with a control group. The low-protein diet, however, was also low in phosphorus, and further experiments showed that the absolute amounts of iron and phosphorus in the diet as well as the iron: phosphorus ratio influenced the extent of iron absorption. They were unable to decide whether proteins or amino-acids might affect iron absorption.

Gillman, Canham and Hathorn (1958) also investigated the relation of poor diet to siderosis in rats. They fed one group uncooked mealie meal (maize meal) and a control group a good stock diet. Four per cent ferric citrate was added to the food of half the rats on each diet. Their results confirmed their previous views, (Gillman et al. 1945; Gillman et al. 1957) that dietary iron overload does not readily induce severe parenchymal cell siderosis if the basal diet is good. Moreover, a poor diet rich in iron produces much more severe siderosis than one low in iron. In later experiments of the same kind (Gillman, Hathorn and Canham, 1959) increased iron absorption was demonstrated by measurement of total body iron. They postulated that dietary siderosis in rats and perhaps in man, too, is attributable to some effects of poor diet on intracellular metabolism. This had also been suggested by Gillman and Gillman (1951), Beutler (1957) and Beutler and Blaisdell (1958).

Rather (1956) and Wohler and Zoll (1960) reported somewhat similar results from their investigations of the effect of protein deficiency on iron absorption and siderosis. Bethard, Wissler, Thomson, Schroeder and Robson (1958) tackled the problem from a different angle. They investigated the effect of protein deprivation on the distribution of an injected tracer dose of radioiron in rat tissues and on erythropoiesis. The protein-deficient rats utilized subnormal amounts of radioiron for erythropoies, and the proportions of the dose found in the liver fluctuated in a characteristic abnormal way. Less radioiron was recovered from the blood and tissues analysed than in normal animals.

These last experiments give no information on the absorption of iron, but the other work cited leaves no room for doubt that iron absorption is increased in protein deficiency particularly when the iron content of the diet is high.

IV. IRON ABSORPTION

After it had become clear that the amount of iron in the body is determined largely by the amount absorbed (the mouse appears to be exceptional, Hampton, 1954), the hypothesis of the "mucosal barrier" was advanced to account for normal and pathological variations in iron absorption (Hahn, Granick, Bale and Michaelis, 1943). According to this hypothesis, the intestinal mucosa contains some barrier to iron absorption which is released when the body requires iron. Granick, (1946) put forward the idea that ferritin might be an obligatory intermediate of iron absorption and that the incorporation of iron into ferritin or its liberation for transfer to the plasma might be the rate limiting step. The theory was later modified in an attempt to account for increased iron absorption in anaemias not associated with iron deficiency (Granick, 1949).

In recent years an irresistible weight of evidence has accumulated that this theory is no longer tenable. Wohler, Heilmeyer, Emrich, and Kang, (1957), and Heilmeyer, Keiderling, and Wohler (1957) have shown that mucosal ferritin metabolism does not limit iron absorption in the way suggested by Granick (1946). The observations described earlier on the relation of protein deficiency to iron absorption are difficult to explain on the "mucosal barrier" hypothesis. The type of block observed in the early experiments of Hahn et al. (1943) cannot explain the lesser absorption of iron by normal than by iron-deficient persons (Brown, Dubach and Moore, 1958). It had also been observed that absorption is increased in several conditions when erythopoiesis is accelerated even when tissue iron stores are high (Moore, 1957). The objections to the theory have been admirably summerized by Brown, Dubach and Moore (1958) Moore (1959 - 60) and Cantrill and Walsh (1962).

Several alternative hypotheses have recently been advanced. Moore (1959 - 60) suggested a hormonal mechanism which has not found experimental support (Krantz, Goldwasser and Jacobson, 1959; Beutler and Buttenwieser, 1960). Brown and Rother (1961) put forward the idea that there might be pathways of iron absorption other than through ferritin. Later Brown and Rother (1963) obtained evidence of the existence of two pathways, a slow one involving protein-bound iron and a so-called "rapid-transit" one possibly involving the amino-acids serine and glycine. They believe that enhanced iron uptake, such as seen in iron deficiency or accelerated erythropoiesis, to be associated with increased participation of the "rapid-transit" mechanism. They considered that passive diffusion might also be involved.

Multiple pathways of absorption have also been suggested by other workers. Bannerman, O'Brien and Witts, (1962) obtained results which might be interpreted on the assumption that simple diffusion and a carrier-assisted process supplemented one another, but they offered no direct evidence. Charlton, Jacobs, Terrance and Bothwell (1963) have produced elegant experimental results which show conclusively that a fraction of absorbed iron passes through ferritin, but that this fraction is small when an iron-deficient subject is absorbing iron avidly. They revive the idea of a controlling function for ferritin, but in a very different way from that originally proposed by Granick (1946). Since increased amounts of orally administered radioiron are incorporated into mucosal ferritin when the need for iron is reduced and less iron is in fact being actually absorbed, they have suggested that this ferritin is lost from the body as the mucosal cells exfoliate. They find it tempting to postulate that ferritin when present in

sufficient concentrations may inhibit the formation of the complex or complexes responsible for active transport through the cells. The mechanism appears inefficient when large quantities of iron are presented to the mucosa, as had also been demonstrated by Wohler *et al.* (1957). It seems that when iron intake is increased above a certain limit, though the mucosal cells become saturated with ferritin, there may still be excess iron available for the formation of the complexes actively transported through the cell.

THE PRESENT PROBLEM OUTLINED

The work described in this thesis was undertaken with two points in mind. First, the desirability of confirming if possible by a different technique, that protein-deficient animals do absorb more iron than normals, and second, the need to have more knowledge of the form in which the excess iron is stored. Ferritin is a protein. Haemosiderin undoubtedly contains protein, but probably stores more iron per gram of protein than ferritin (Behren and Asher, 1933; Asher, 1933; Ludewig, 1957; McKay and Fineberg, 1958; Richter, 1960; Shoden and Sturgeon, 1960). Many proteins e.g. haemoglobin, plasma proteins, muscle proteins, are synthesized in subnormal quantities in protein deficiency. The fundamental importance of the relation of excess iron storage to protein deficiency needs no further emphasis.

A series of experiments has been done in which groups of rats kept on normal and protein-deficient diets have been tested at intervals for their ability to absorb iron from a standard dose. Other animals on similar dietary regimes have been killed and the livers and spleens analysed for iron present as ferritin and as haemosiderin. It was found convenient to make observations on haemoglobin, serum iron, and iron binding capacity and on packed cell volume when the rats were killed. The protein-deficient diet was usually protein-free, but in one experiment the effect of including zein in the diet was studied.

Table 1

COMPOSITION OF SYNTHETIC DIETS (Gm. Per 100 Gm.)

	A Casein Diet	B Zein Diet	C Protein Deficient Diet
DEXTROSE	48.8	48.8	70.8
CASEIN	22	–	–
ZEIN	–	22	–
CORN OIL	4	4	4
AGAR	5	5	5
★SALT MIXTURE	4	4	4
☆MULTI-VITAMIN MIXTURE	2.2	2.2	2.2
WATER	14	14	14

★ U.S.P., XV, 883, 1955.

☆ N.B.Co. 1962.

EXPERIMENTAL

Animals

Adult male Wistar-strain albino rats were put in groups of six or eight in metabolic cages but with no precautions against coprophagy. From the amount of faeces recovered daily this seems to be too small to affect the investigation.

In some of the experiments young rats weighing between 87 and 197 grams (average 147 grams) were used. In other experiments bigger rats weighing between 250 and 395 grams (average 320 grams) were used.

In the first two experiments the animals were examined and weighed twice weekly. Later, weekly weighing was found sufficient. Any rats showing signs of infection were discarded. In fact throughout the work only eight rats were excluded from consideration. Seven of these were protein-deficient.

On reviewing the literature one finds great variation in the amounts of storage iron in the different groups of rats used by different workers. Due to these variations it was found necessary in each experiment to use rats from the same litter and breed, and brought up under the same environmental and dietary conditions, so that they would be of the same age and of comparable weights.

TABLE 2

COMPOSITION OF THE SALT MIXTURE

U.S.P. (1955) XV, 883

Sodium Chloride	139.3 grams	Manganese Sulphate	4.01 grams
Potassium Dihydrogen Phosphate	389.0 "	Zinc Sulphate	0.548 grams
Magnesium Sulphate	57.3 "	Potassium Iodide	0.97 grams
Calcium Carbonate	381.4 "	Cupric Sulphate	0.477 grams
Ferrous Sulphate	27.0 "	Cobaltous Chloride	0.023 grams

TABLE 3

VITAMIN DIET FORTIFICATION MIXTURE IN DEXTROSE

N. B. Co. 1962

	g./100 lbs. diet
Vitamin A Concentrate (200, 000 units per gram)	4.5
Vitamin D Concentrate (400, 000 units per gram)	0.25
Alpha Tocopherol	5.0
Ascorbic Acid	45.0
Inositol	5.0
Choline Chloride	75.0
Menadione	2.25

	g./100 lbs. diet
p Aminobenzoic Acid	5.0
Niacin	4.5
Riboflavin	1.0
Pyridoxine Hydrochloride	1.0
Thiamine Hydrochloride	1.0
Calcium Pantothenate	3.0

	mg./100 lbs. diet
Biotin	20
Folic Acid	90
Vitamin B_{12}	1.35

1 kilogram is sufficient for 100 lbs. diet

Diets

The composition of the synthetic diets fed to the rats in the various experiments is shown in Tables 1, 2 and 3. The three diets were isocaloric, and of the same composition except for the quantity and quality of the protein fraction. Each rat was given 20 grams of the appropriate diet per day, but the protein-deficient rats, after about 3 weeks on the diet, consumed only 15 grams per rat per day, while the rats on the other diets consumed 20 grams each per day.

Duration of Experiments

The animals were kept on the experimental diets for periods up to eleven weeks. Absorption tests were carried out after three, five, six, seven and eight weeks. Later observations were not possible because the passage of a tube into the stomach became very difficult in the protein-deficient rats. For tissue analysis several animals were taken from each group at weekly intervals.

It is interesting to note that although the protein-deficient rats lost weight continuously and became sluggish and of unhealthy appearance, very few of them actually died.

Fig 1

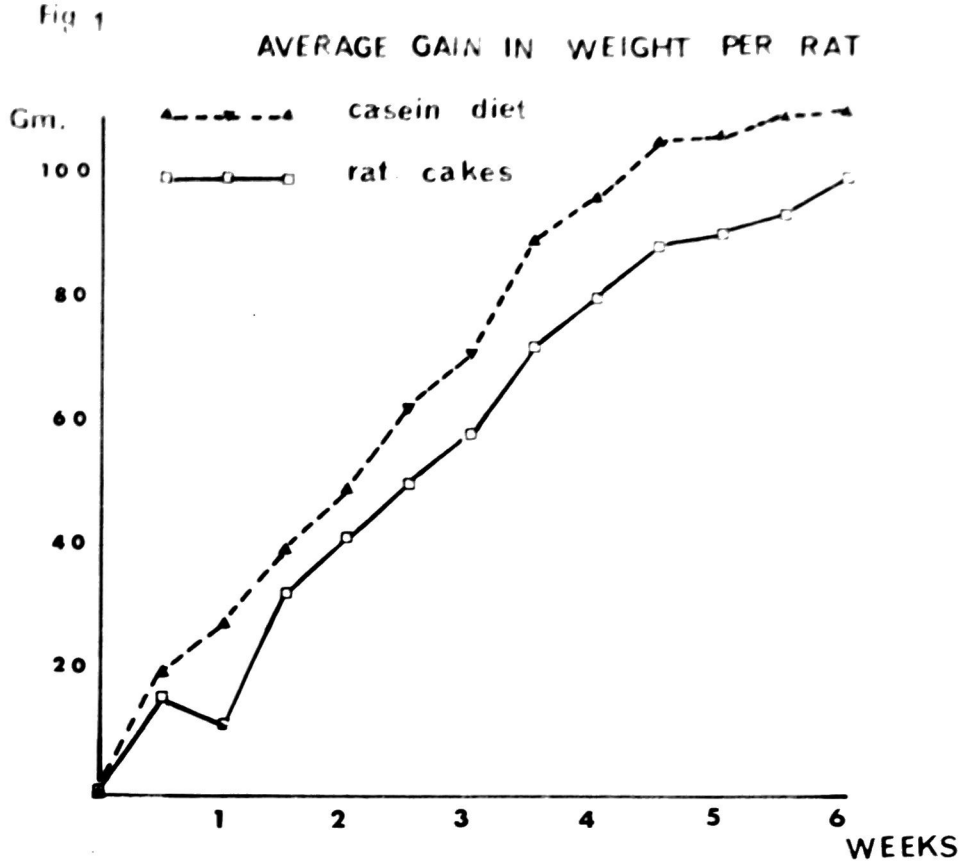

AVERAGE GAIN IN WEIGHT PER RAT
Gm.
casein diet
rat cakes
100
80
60
40
20
1
2
3
4
5
6
WEEKS

Fig.2

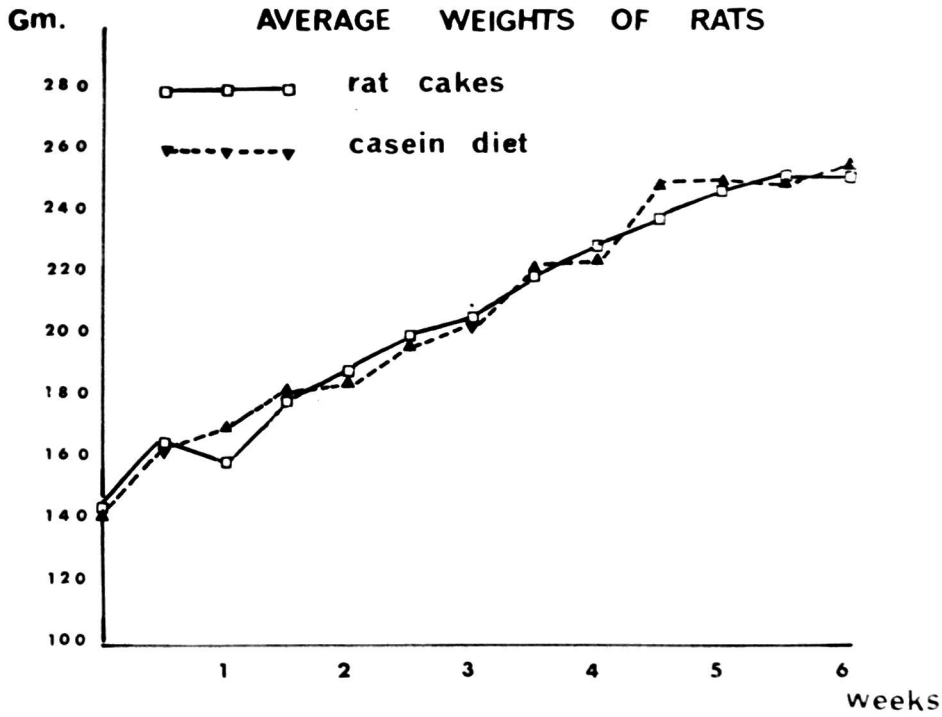

Gm.
AVERAGE WEIGHTS OF RATS
rat cakes
casein diet
280
260
240
220
200
180
160
140
120
100
1
2
3
4
5
6
weeks

Fig. 3

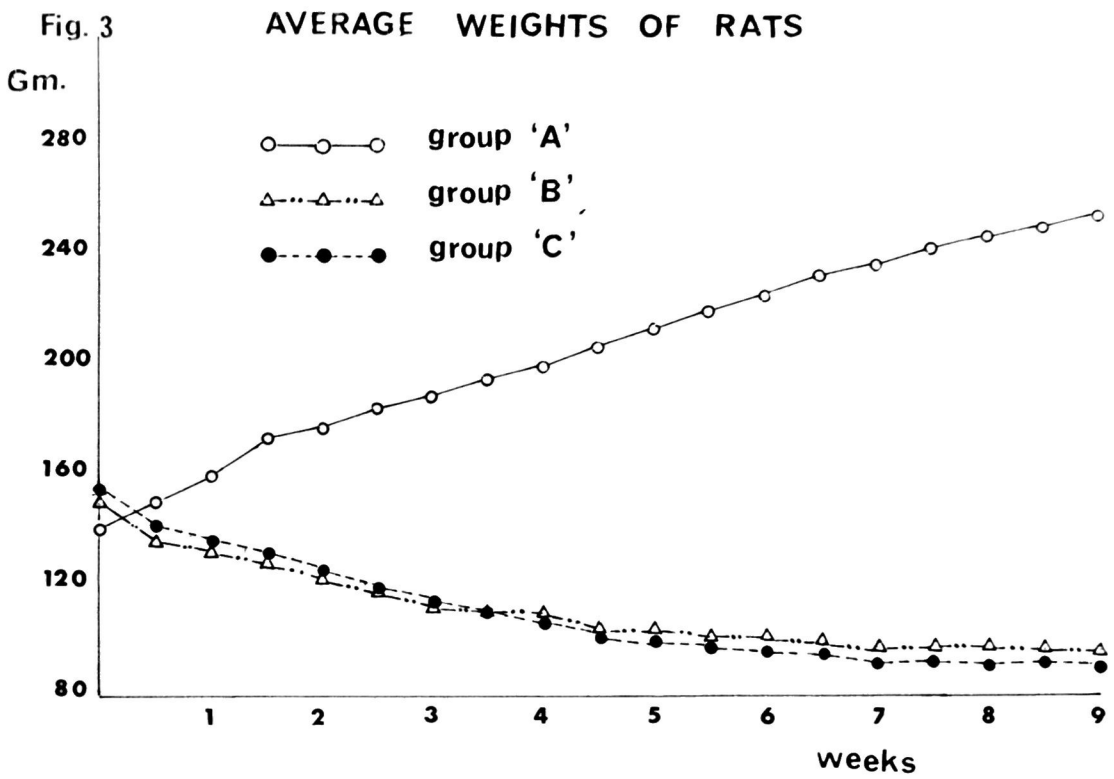
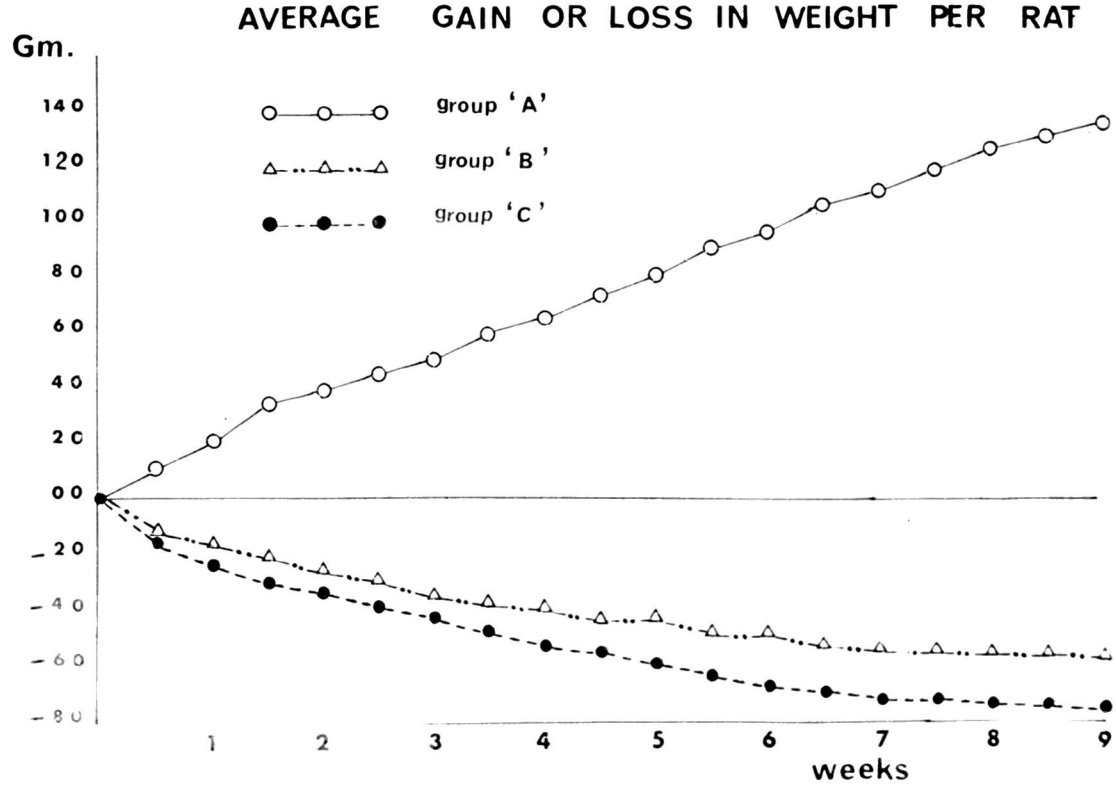

AVERAGE WEIGHTS OF RATS
Gm.
280
240
200
160
120
80
group 'A'
group 'B'
group 'C'
1
2
3
4
5
6
7
8
9
weeks

Fig. 4


AVERAGE GAIN OR LOSS IN WEIGHT PER RAT
Gm.
140
120
100
80
60
40
20
00
-20
-40
-60
-80
group 'A'
group 'B'
group 'C'
1
2
3
4
5
6
7
8
9
weeks

PRELIMINARY OBSERVATIONS

Weights

It was observed that the rats on the stock diet and those on protein-adequate casein diet "A" (Table 1) gained weight steadily and at the same rate. After six weeks each rat on the stock diet had gained an average of 100 grams and each rat on the casein diet "A" gained an average of 110 grams (Fig. 1 and 2). On the other hand the rats fed the zein diet "B" lost weight (58 grams in nine weeks). Those fed the protein free diet "C" lost even more (75 grams in nine weeks). While the numbers of rats in the groups were too small by the end of the experiment for statistical analysis, the losses in weight at six weeks were highly significant: group B (9 rats) had lost 48 grams (S.e $\pm$ 1.0) and group C, 68 grams (S.e. $\pm$ 2.0). The difference of 20 grams with a standard error of 2.3 is obviously highly significant. These results are given graphically for the whole period in Figs. 3 and 4.

FIG. 5

Average Weights of Rats.

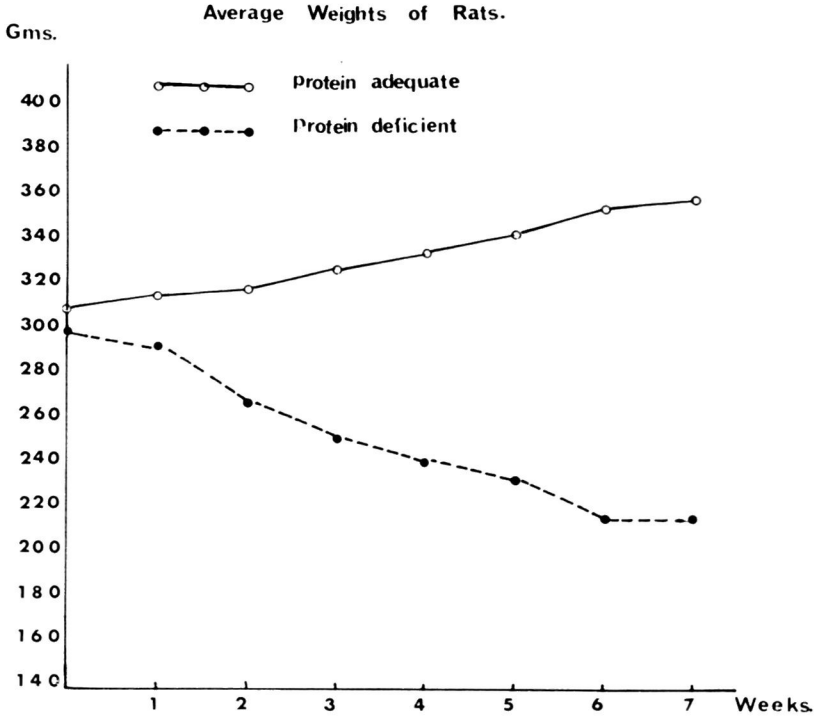


FIG. 6

Average Gain or Loss in Weight per Rat

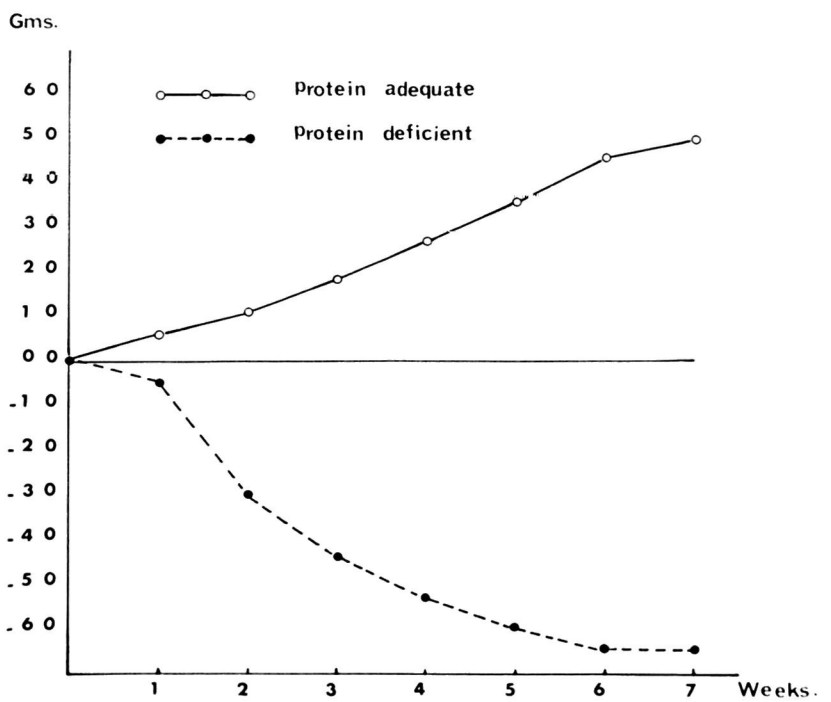


Fig. 7 AVERAGE WEIGHTS OF RATS

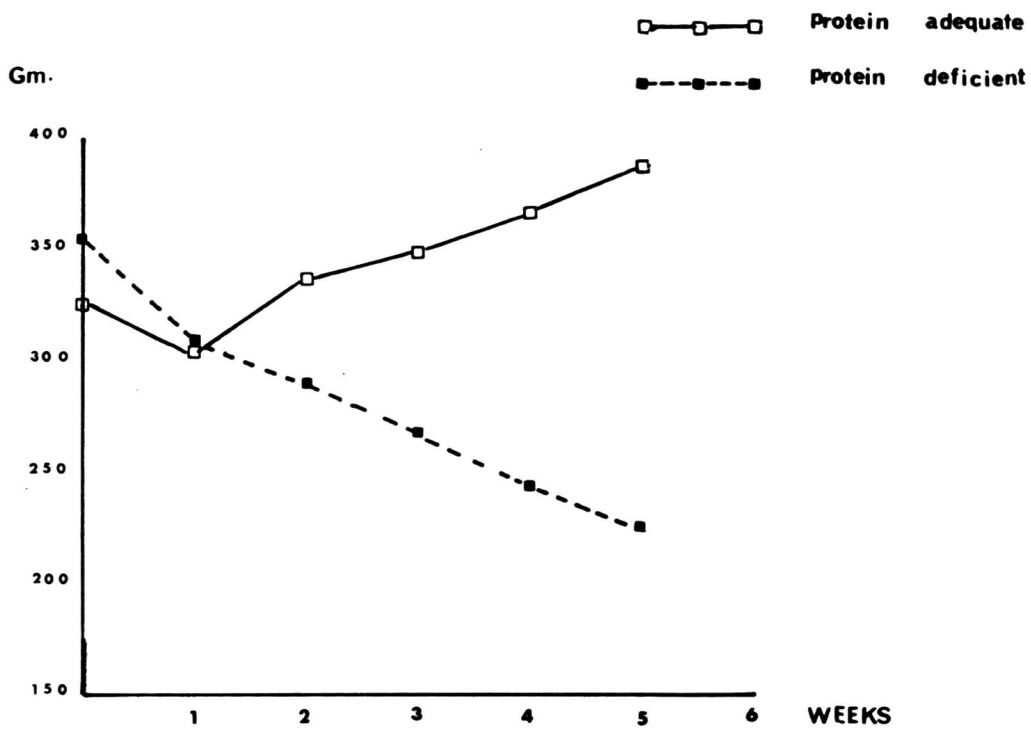
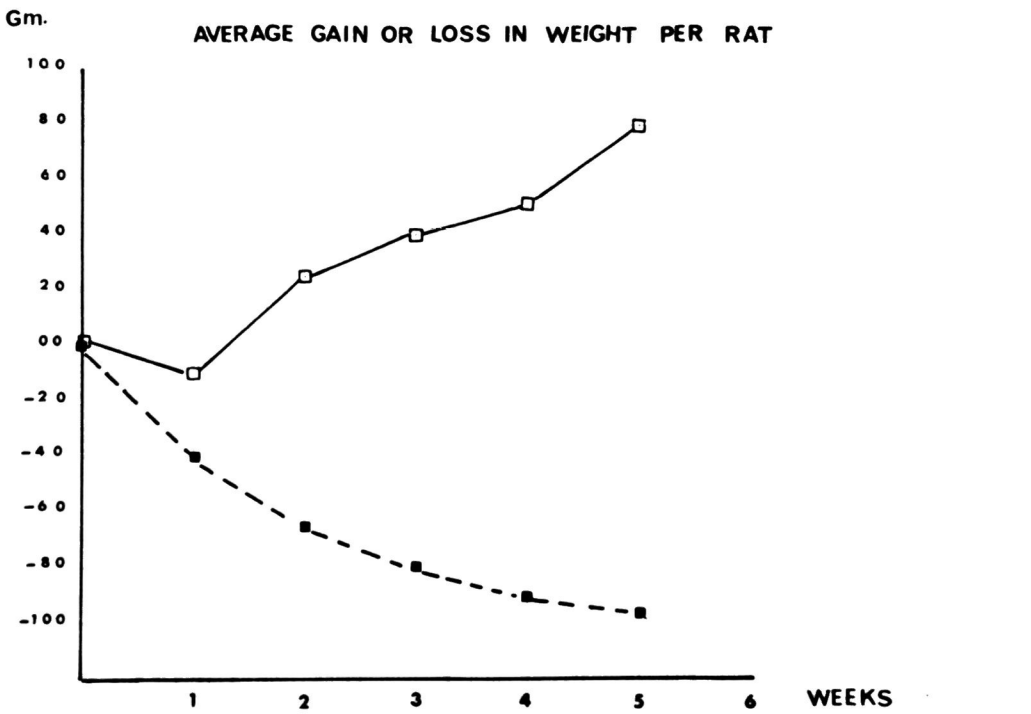


Fig. 8



Looking at Figs. 5 and 6 it becomes evident that the big rats on casein diet "A" did not gain as much weight as the small rats fed the same diet, 50 grams as compared with 105 grams per rat, in the same period of seven weeks. However, the big rats on protein-free diet lost weight at the same rate as the small rats on the same diet, about 65 grams as compared with 70 grams per rat in seven weeks. When excess iron was added to the diets, the weights of the rats showed the usual pattern with slight exaggeration. The rate of weight gain by the protein-adequate rats and the rate of weight loss by the protein-deficient rats were both greater than in the rats of the same initial weight group but given normal iron in their diets (Figs. 7 and 8).

Blood Analysis

When specimens were required for blood analysis and for the other analytical observations, the animals were anaesthetized with ether. Blood was taken by cardiac puncture in a heparinized syringe for haemoglobin, serum iron, total iron bind capacity, and packed cell volume determinations.

Fig 9

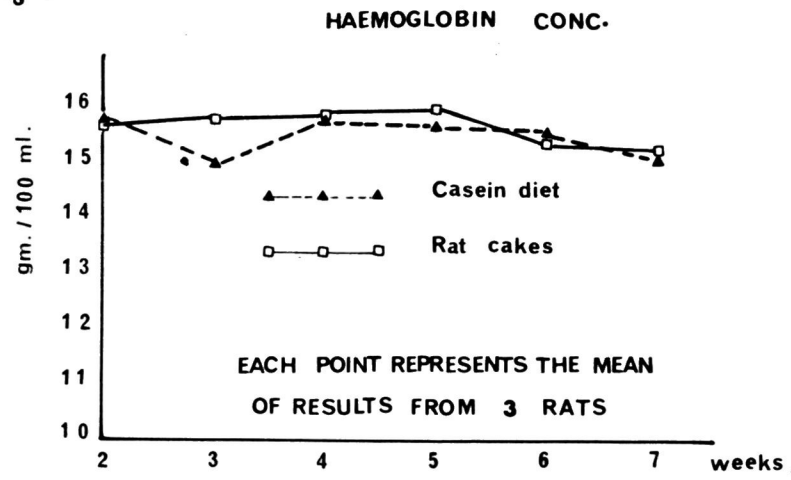

HAEMOGLOBIN CONC.
gm./100 ml.
16
15
14
13
12
11
10
Casein diet
Rat cakes
EACH POINT REPRESENTS THE MEAN
OF RESULTS FROM 3 RATS
2
3
4
5
6
7
weeks.

fig. 10

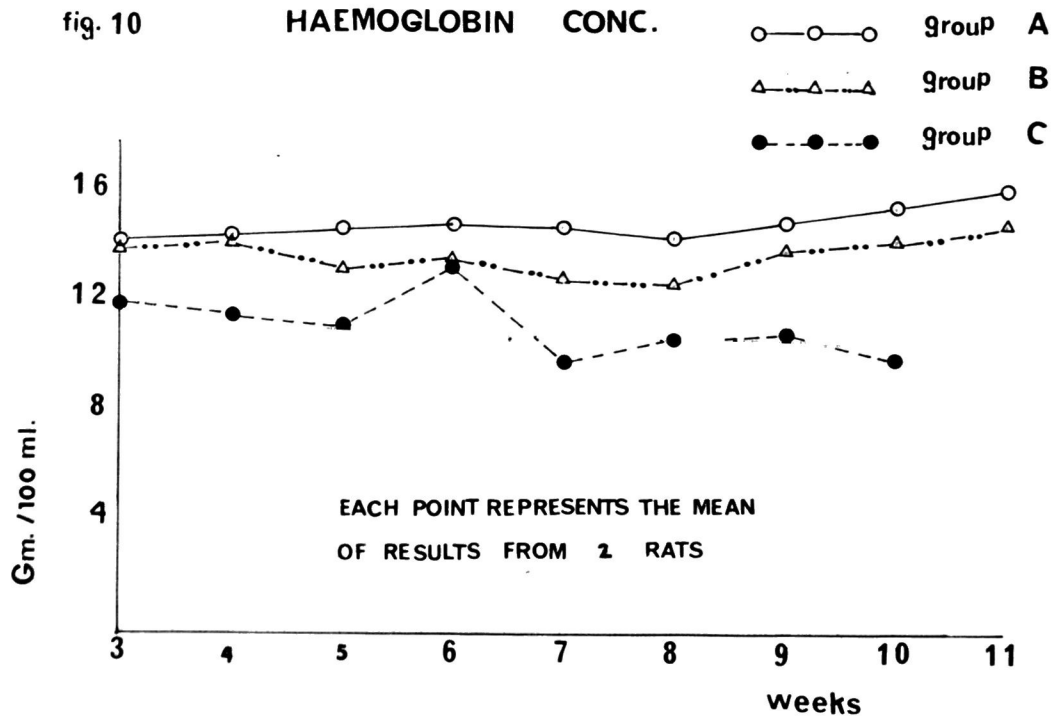
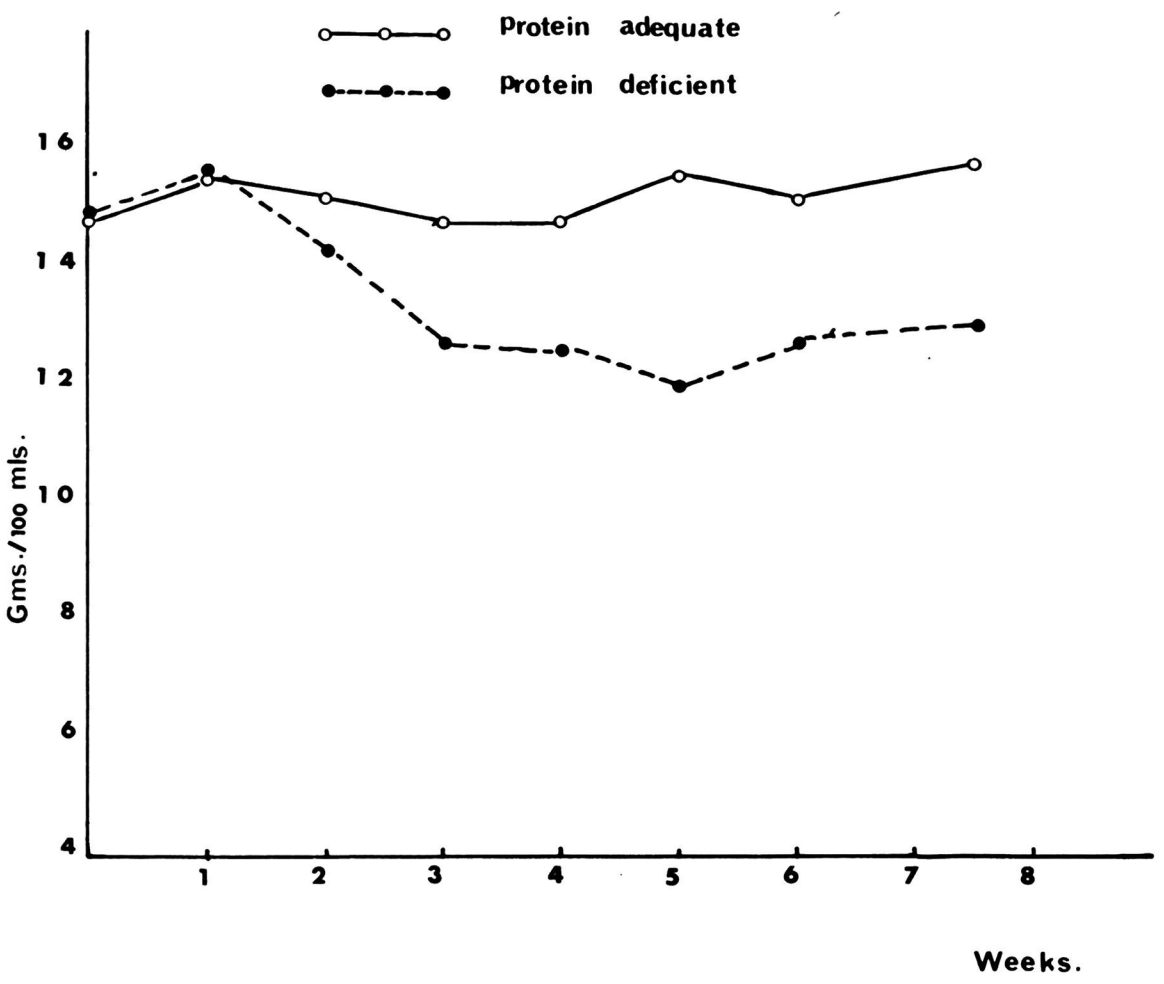

HAEMOGLOBIN CONC.
group A
group B
group C
Gm./100 ml.
16
12
8
4
EACH POINT REPRESENTS THE MEAN
OF RESULTS FROM 2 RATS
3
4
5
6
7
8
9
10
11
weeks

FIG.11

Hæmoglobin Concentration.

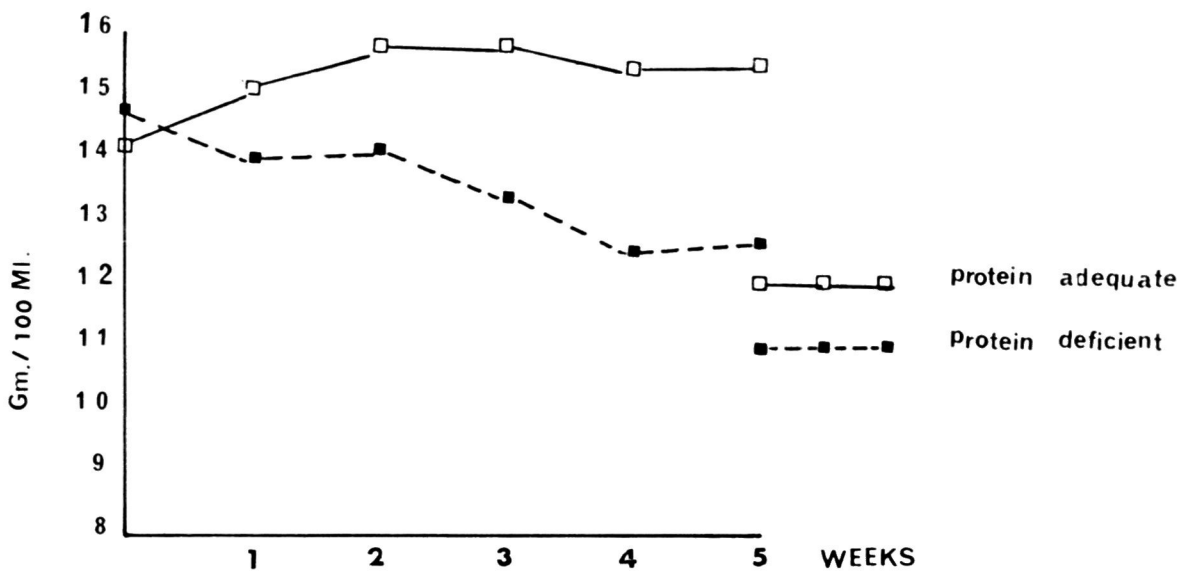


☆ Each Point is an average of 6 rats.

fig. 12

HAEMOGLOBIN CONC.

Each Point is mean of 6 rats



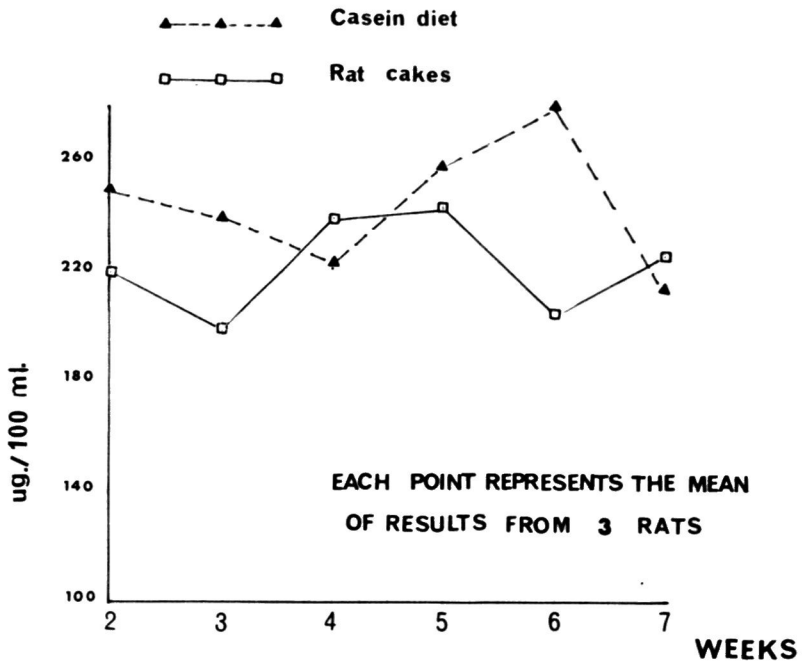
Haemoglobin

The haemoglobin concentration was calculated from the total blood iron determination (Ramsay, 1964).

In the rats fed the stock diet and those fed the synthetic casein diet "A", the haemoglobin remained constant atabout 15 - 16 grams/100 ml. (Fig. 9). In the rats fed the zein diet "B" it showed a slight drop to range from 13 to 15 grams/100 ml. (Fig. 10). The maximum drop, however, appeared in the rats fed the protein-free diet "C" where it ranged from 10 to 13 grams/100 ml. depending on the duration of the experiment (Fig. 10 and 11). Addition of excess iron to the diets did not have any significant effect on these ranges (Fig. 12).

fig. 13

SERUM IRON



SERUM IRON

fig. 14

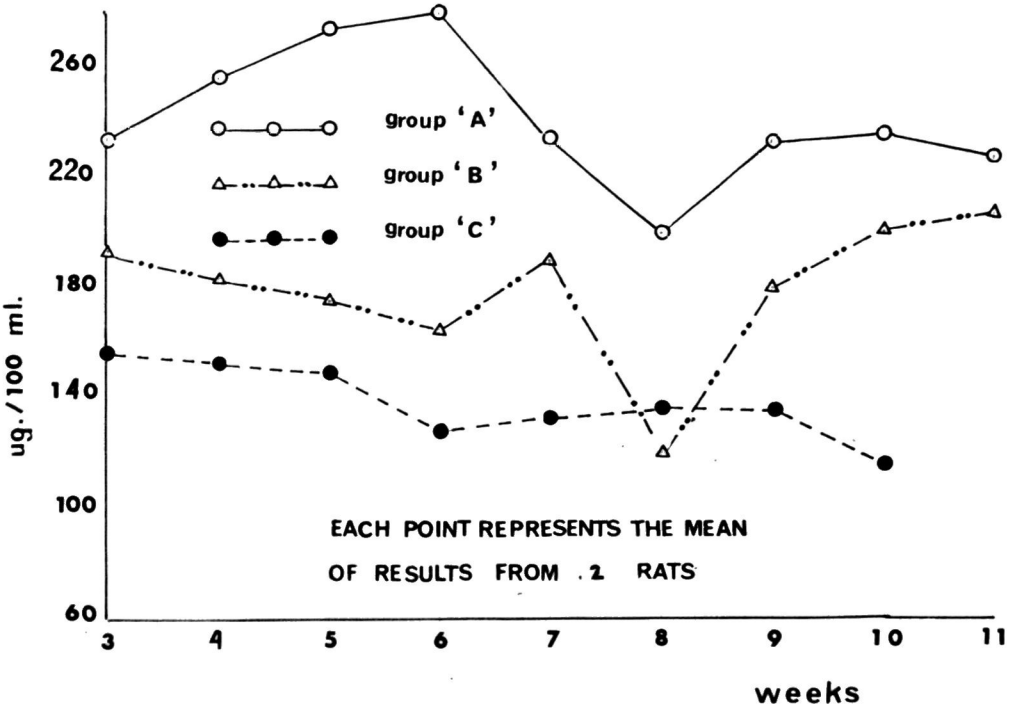
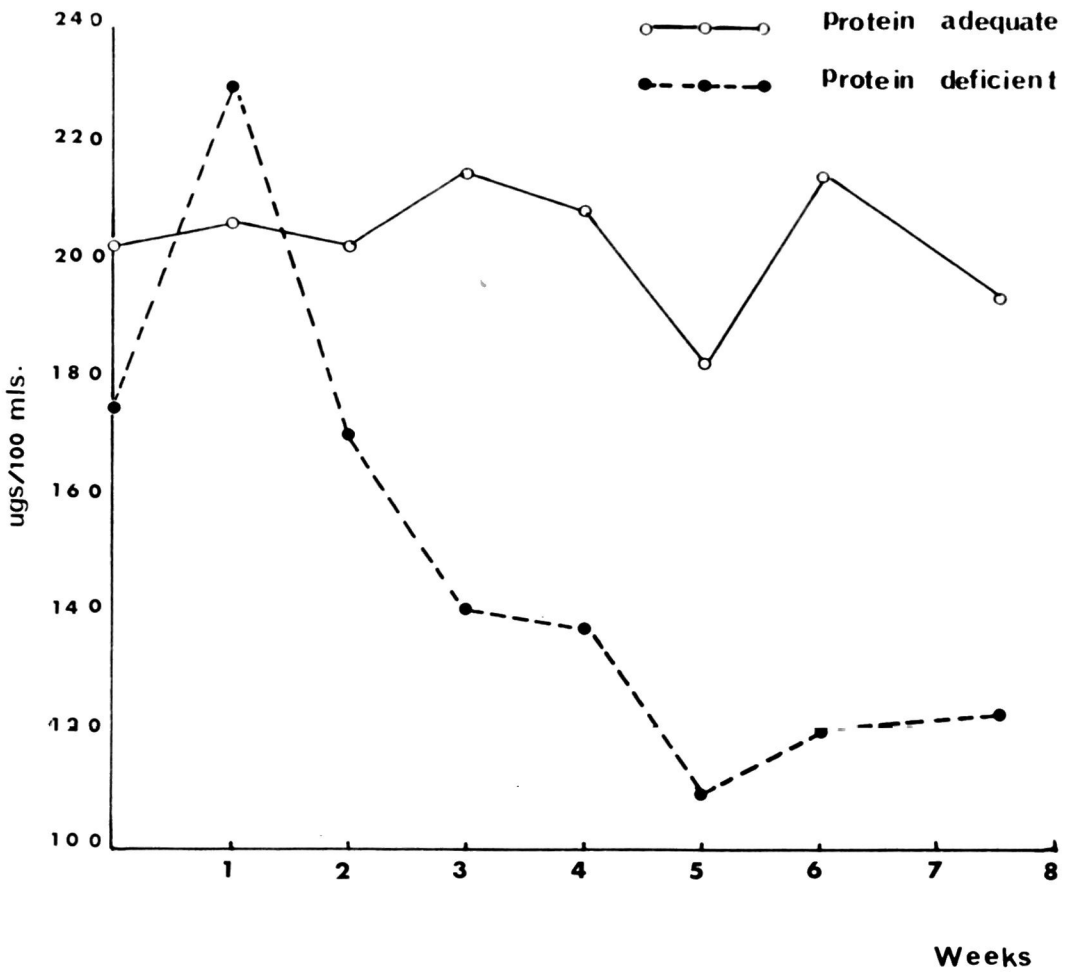


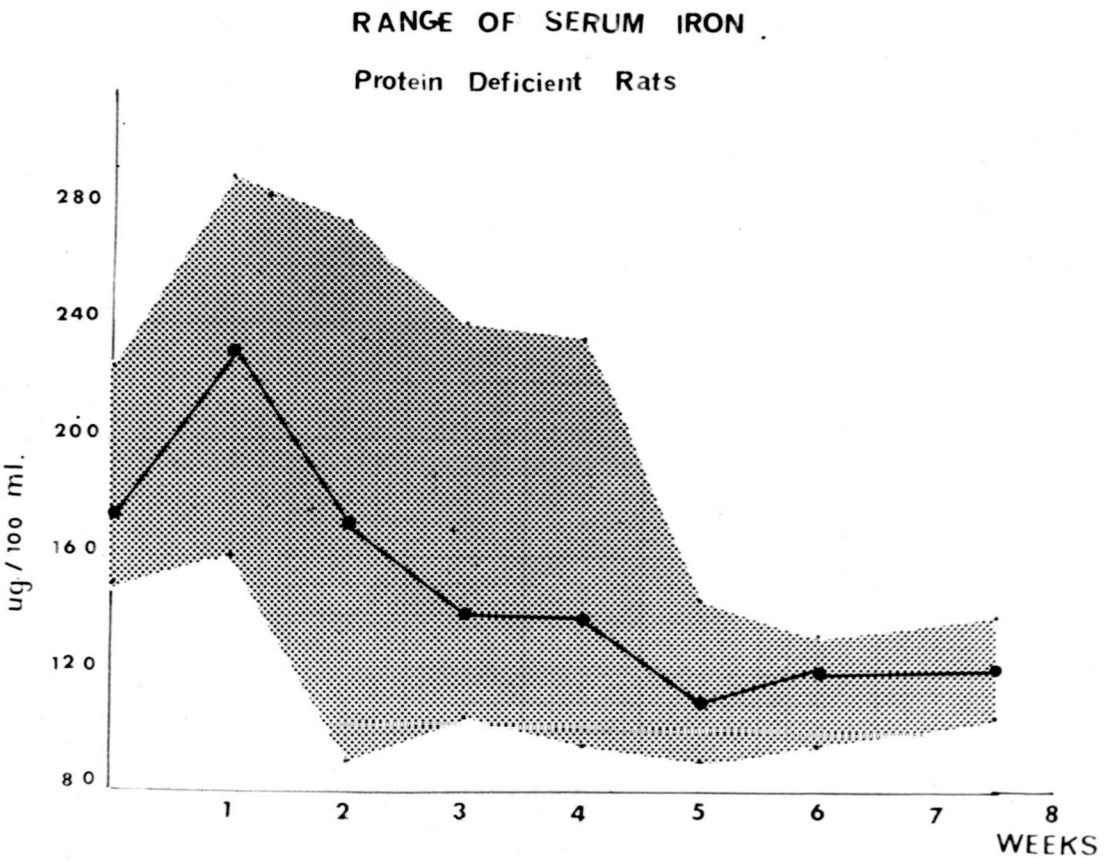
FIG. 15

SERUM IRON



☆ Each point is an average of 6 rats

Fjg. **15 a**

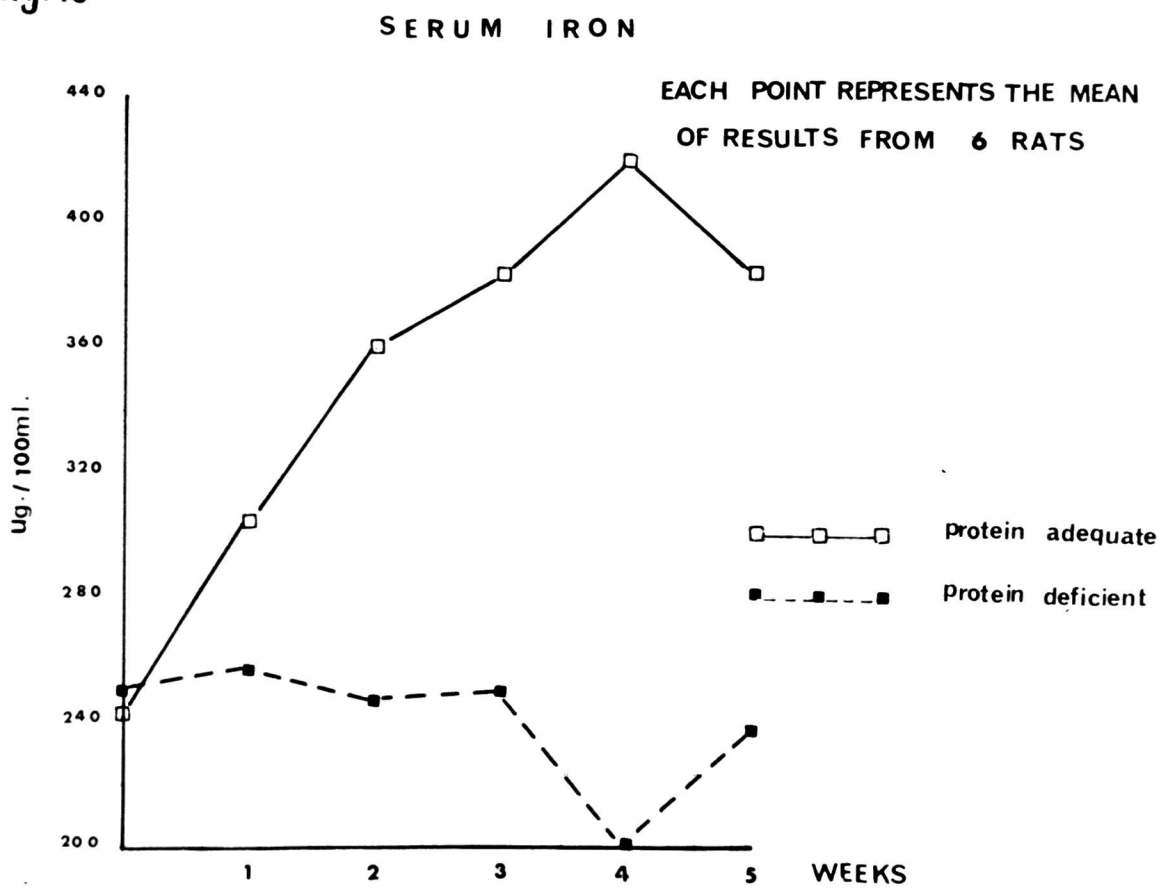

RANGE OF SERUM IRON
Protein Deficient Rats
280
240
200
160
120
80
ug / 100 ml.
1
2
3
4
5
6
7
8
WEEKS

Serum Iron

In the normal rats fed the stock diet and those fed the casein diet "A" the serum iron (Ramsay, 1957 a) showed the usual variation from rat to rat but stayed within the normal range of 200 - 280 µg./ml. (Fig. 13). In the rats fed the protein-free diet "C" there was a definite drop to a low level of 130 µg./100 ml. (Fig. 14 and 15), while in the rats on the zein diet "B" it was intermediate between the values in the normal and protein-deficient rats (Fig. 14).

In the rats fed diet "C" the serum iron rose to about 230 µg./100 ml. in the first week but on plotting all the six points (Fig. 15 a) there was a large scatter round the mean which would suggest the insignificance of this apparent rise, though it ties well with the idea of haemoconcentration suggested by Bethard et al. (1958).

fig. 16

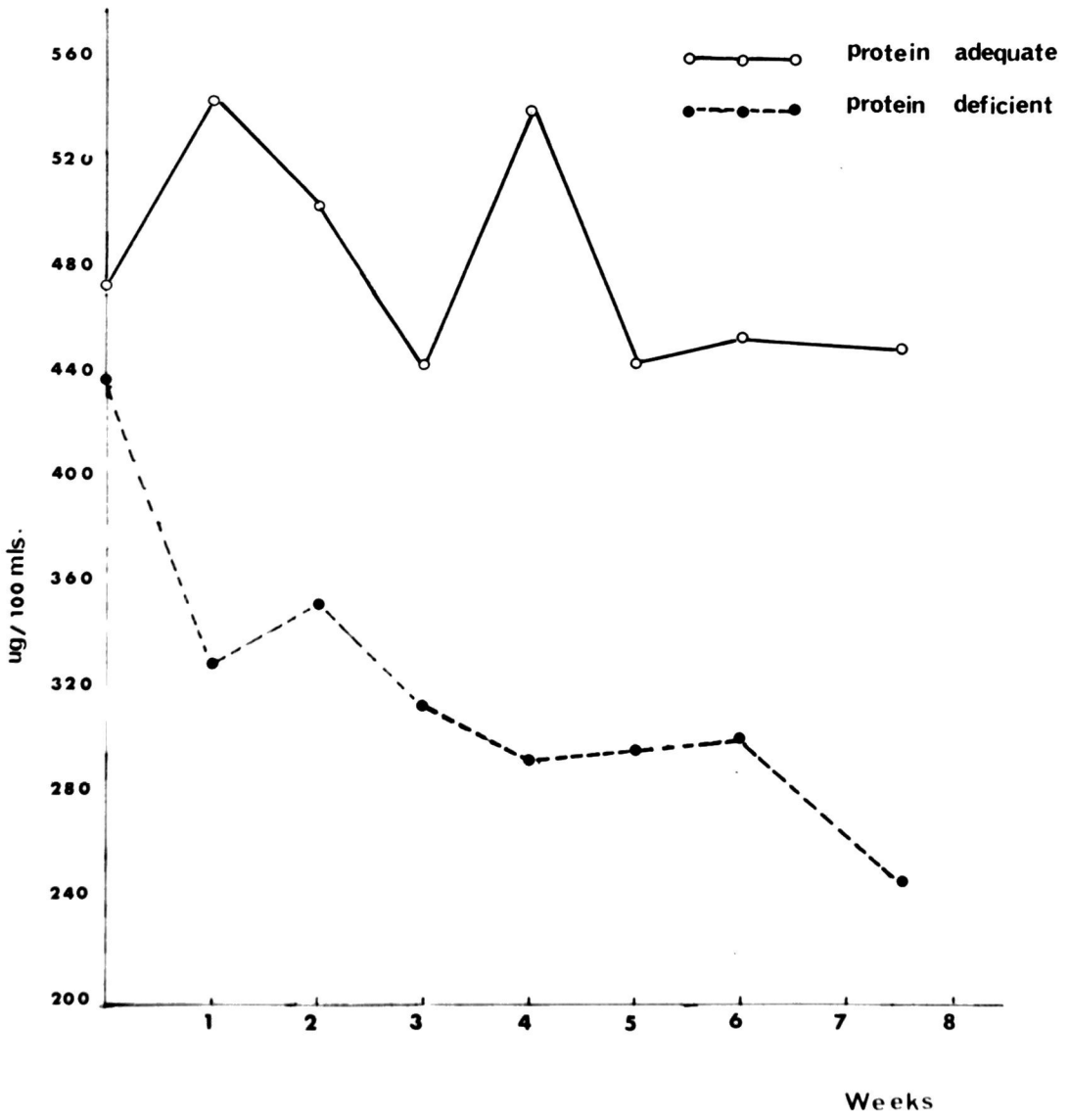

SERUM IRON
EACH POINT REPRESENTS THE MEAN
OF RESULTS FROM 6 RATS
440
400
360
320
280
240
200
ug./100ml.
1
2
3
4
5
WEEKS
Protein adequate
Protein deficient

It is interesting to note that the haemoglobin concentration in the same rats reached its highest level in that week (Fig. 11).

When excess iron was added to the diets, the serum iron of the rats fed diet "A" rose to above 400 µg./100 ml. while in the rats fed the protein-free diet "C" it remained at a constant level of about 240 µg./100 ml. (Fig. 16). Thus, the excess iron intake appeared to prevent the drop of serum iron level observed in protein-deficient rats given normal iron (Figs. 14 and 15), although the increase observed in the corresponding group of protein-adequate rats was not seen.

Fig. 17

TOTAL IRON BINDING CAPACITY



☆ Each Point is an average of 6 rats.

Total Iron Binding Capacity

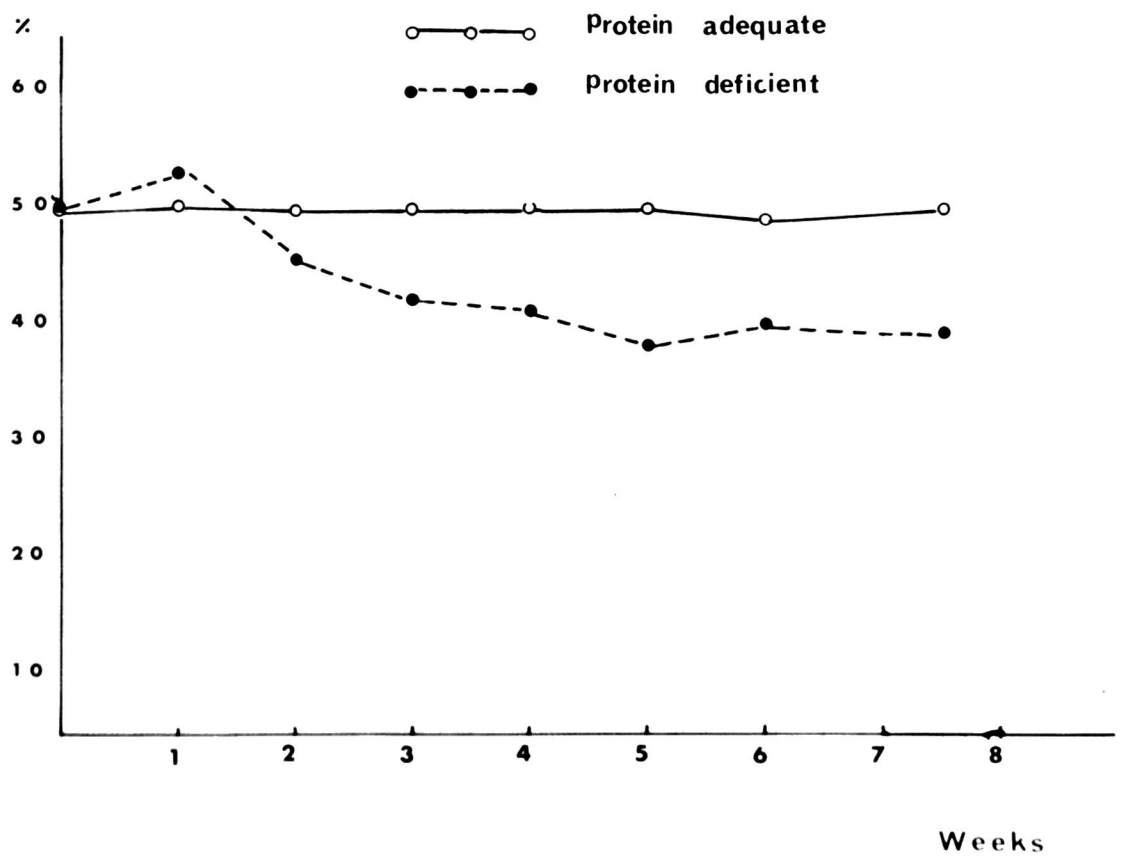
The average total iron binding capacity of plasma (Ramsay 1957 b) of the rats on the casein diet "A" fluctuated between 440 and 540 µg./100 ml. in the course of the experiment while in the protein-deficient rats it showed a marked drop to just above 240 µg./100 ml. at the end of the experiment (Fig. 17). There was, however, no rise in the first week which is against the suggestion of haemoconcentration to which reference was made on page 29.

Packed Cell Volume

The packed cell volume was measured using Wintrobe haematocrit tubes. In the rats on diet "A" it remained constant at approximately 50%, while in the protein-deficient rats it dropped to 40% (Fig. 18), although at the end of the first week the value rose to 53%. The value for each of the six rats analysed that week was 52% or above.

Fig.18

PACKED CELL VOLUME



☆ Each point is an average of 6 rats

Whether this indicated haeme concentration, one could not be sure from one set of results.

It is interesting to follow the sequence of changes occurring in haemoglobin, serum iron, total iron binding capacity and packed cell volume in the protein-deficient rats.

In the first week there was considerable fluctuation and, if anything, the mean values rose above normal except the total iron binding capacity which actually decreased.

For three or four weeks the values showed gradual decrease, and then remained approximately constant with very slight change. The total iron binding capacity, however, showed continuous fall throughout the experiment.

IRON ABSORPTION

Estimation of the total body-iron was used as a measure of long term absorption in small experimental animals by Gillman et al. (1959), but the long elaborate procedures involved in the process of ashing and iron estimation, especially when a large number of animals would need to be analysed, makes it unsuitable for our purpose if simpler and more direct methods are available. The recent development of whole body counters provides an accurate device for studying the retention of an orally administered dose of radioiron (Field, Seki, Mitchell and Chalmers, 1960; Forrester, Conrad and Crosby, 1962; Veall and Vetter, 1958; Bannerman et al. 1962; Gitlin and Crushaud, 1962; Price, Cohn, Wasserman, Reizenstein and Cronkite, 1962). The fact that the excretion of iron certainly over a period as short as a few days, is negligible means that retention is equivalent to absorption. This method has the advantage that the capacity to absorb iron from a standard dose can be tested at any time during the course of the experiment. It has been used in the present investigation.

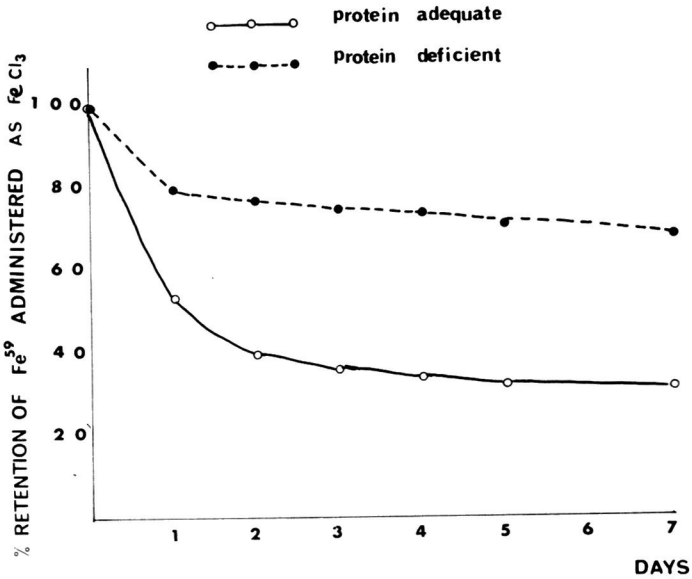
APPARATUS AND PROCEDURE

The apparatus used is a multi-tube gamma ray counter, a modified version of that described by Veall and Vetter, (1958). It consists of 12 large counter tubes (Type G26 Pb) connected in parallel and symmetrically disposed around a suitably large central container which can accommodate a rat-restriction cage. An outer casing provides protection, shielding from light, and electrical screening for the counter tubes. A further outer container giving an annular space one and a half inches wide is filled with lead shot for shielding. The background count was 1200 c.p.m. and 1 uc. ^{59}Fe (as $FeCl_3$) gave an additional 10,000 c.p.m.

The rats were fasted for 16 hr. except for free excess to 5% glucose in distilled water. Under light ether anaesthesia a stomach tube was passed and each rat was given 1 ml. of radioiron-ascorbic acid solution containing 1 uc. of ^{59}Fe as ferric chloride with measured amounts of ferric chloride carrier and ascorbic acid mixed prior to administration. The rats were counted immediately and then daily for seven days. The percentage retention of the administered dose was then plotted against time.

Fig. 19

RADIOIRON ABSORPTION
BY TOTAL BODY COUNTING

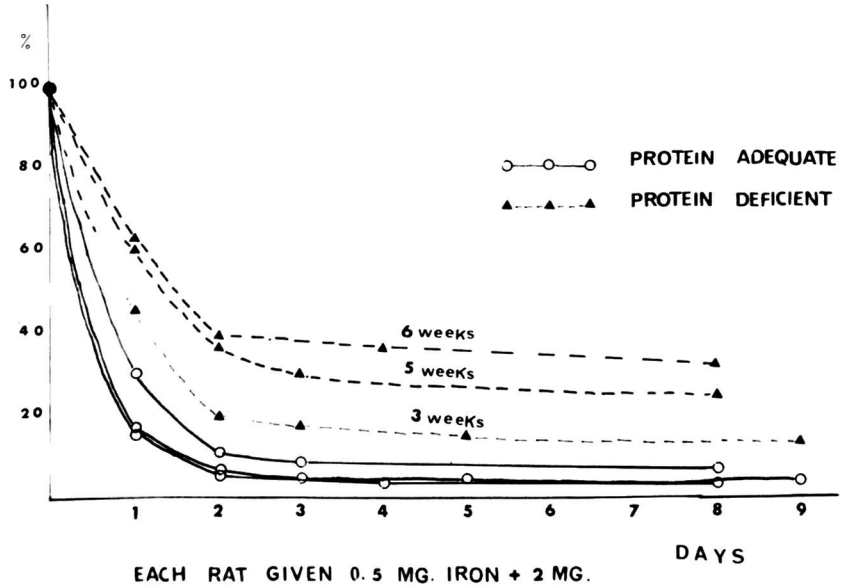


EACH RAT GIVEN 0.2 MG. OF IRON+2 MGS. OF ASCORBIC ACID.

☆ EACH POINT IS AN AVERAGE OF 12 RATS

Fig. 20

PERCENTAGE RETENTION OF Fe^{59} AS $FeCl_3$



EACH RAT GIVEN 0.5 MG. IRON + 2 MG.
ASCORBIC ACID

PRELIMINARY EXPERIMENTS

A. After seven weeks on the diets, 12 protein-adequate rats and 12 protein-deficient rats were given by stomach tube the radioiron-ascorbic acid solution containing 0.2 mg. of iron and 2 mg. of ascorbic acid.

It was observed that, although there was considerable variation among individual rats, more than half the counts were lost in the first day, followed by another loss on the second day, and insignificant amounts were lost on subsequent days (Fig. 19). This observation agrees with the earlier radioiron absorption studies of Austoni and Greenberg (1940), who found that in the rat the greater part of faecal loss of radioiron took place within 48 hr.

After seven days the average percentage retention of radioiron per rat was 31% and 68% in the normal and protein-deficient rats respectively (Fig. 19).

TABLE 4

Percentage retention of graded doses of radioiron and ascorbic acid by normal rats.

No. of Rats	Iron fed (mg.)	Ascorbic acid (mg.)	Percentage ^{59}Fe Retained	
			Individual rats	Mean
3	2	4	2.0, 0.9, 1.0	1.3%
6	1	2.5	2.8,2.3,2.4,1.9, 1.2, 1.2	2%
4	0.5	2.0	5.5, 3, 3.8,1.7	3.5%
4	0.2	1.0	15.5, 10, 14, 13	13.1%

In view of the fact that some of the protein-deficient and normal rats retained nearly 90% of the administered dose, it was thought desirable to try to find a dose of iron which would be unlikely to give a high degree of absorption in normal animals and which would yet be so small that protein-deficient rats might absorb a substantial proportion of it.

B. Dose Selecting Experiment

Groups of normal rats were given by stomach tube graded amounts of radioiron with a modest excess of ascorbic acid (1.3 - 1.6 moles/gram atom iron). The percentage retention of the administered doses of radioiron was determined as before (Table 4). 0.5 mg. radioiron with 2 mg. ascorbic acid was then chosen as the standard dose for the following experiments.

EXPERIMENT I

Rats weighing between 250 and 360 grams (average 305 grams) were divided into two groups. One group was fed the casein diet "A" and the other was given protein-free diet C (see Table 1). After three weeks, six rats from each group were given the radioiron-ascorbic acid solution by stomach tube and counted immediately and then daily for a week. The same procedure was repeated on similar groups of rats after five and six weeks on the diets.

TABLE 5

Radioiron retention by normal and protein-deficient rats.

No. of rats	Diet	Weeks on Diet	Percentage retention of ^{59}Fe	
			Individual Rats	Mean
6	Casein "A"	3	3.5, 4.0, 3.8, 3.4, 3.2, 3.9	3.6%
6	Protein Free "C"	3	10, 4.7, 8.2, 4.0, 10.5, 39.6	12.8%
6	Casein "A"	5	5.3, 9.4, 6.3, 9.3, 5.7, 5.5	6.9%
6	Protein Free "C"	5	10, 33, 11.6, 36.7, 37.2, 16.9	24.2%
5	Casein "A"	6	4.6, 4.1, 3.7, 3.0, 2.8	3.6%
4	Protein Free "C"	6	17.9, 34.6, 61.7, 15.2	32.4%

Each rat was given 0.5 mg. ferric chloride carrier, 2 mg. ascorbic acid with 1 μc. ^{59}Fe.

Results and observations

The results are summarized in Fig. 20. The normal control rats retained an average of 3.6%, 6.9% and 3.6% after 3, 5 and 6 weeks on the diet respectively. The corresponding averages for the protein-deficient rats were 13%, 24% and 32%.

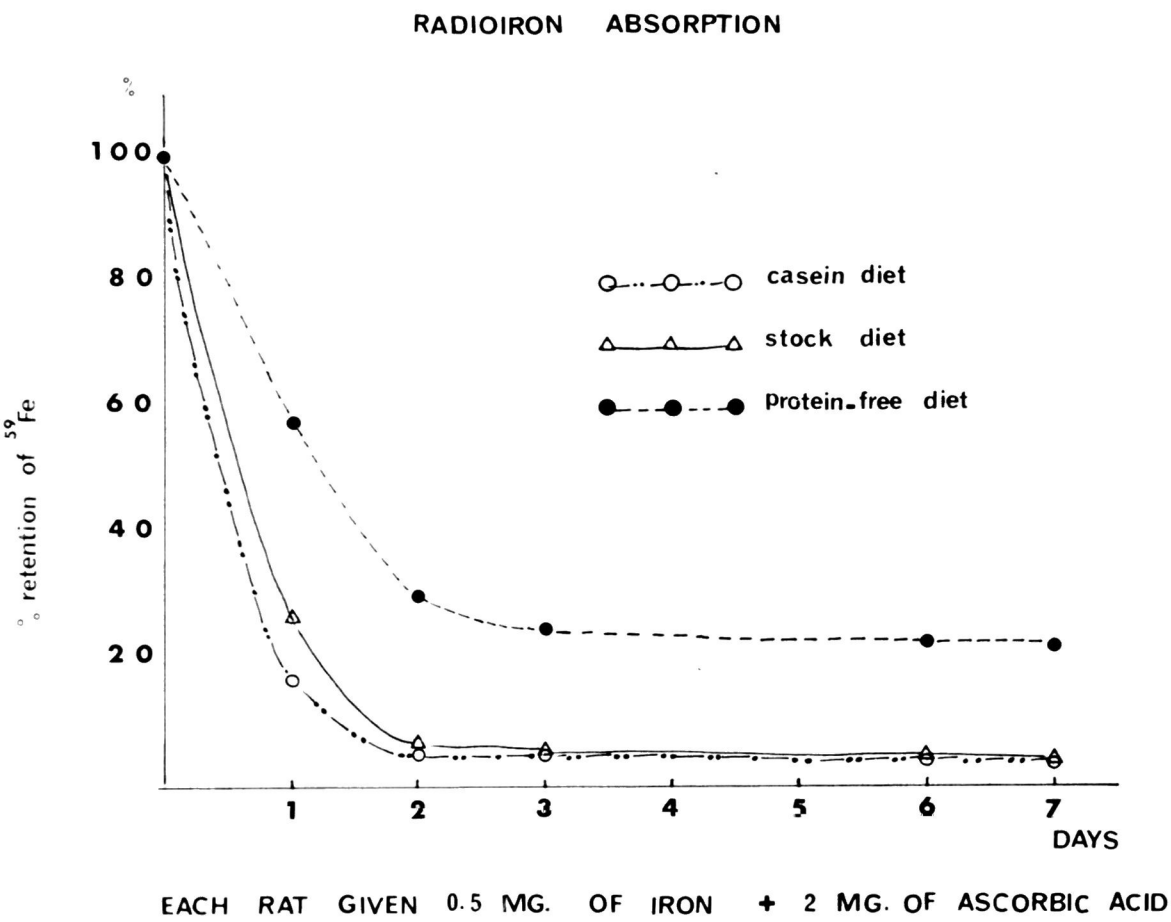
It can be seen that the percentage retention of radioiron administered to normal rats remained low through-the experiment, while the protein-deficient rats appeared to retain progressively more as they became more protein-deficient. Consideration of the figures for individual rats reveals considerable variation among the rats on the protein-free diet (Table 5).

The earlier observation that the bulk of faecal loss of radioiron occurring in the first 48 hr. was also observed in this experiment in both groups of rats.

EXPERIMENT II

As casein is a phosphoprotein, and the amount of phosphate in the diet is known to affect iron absorption (Hegsted et al. 1949) it seemed desirable to compare the effect of the casein diet with that of the laboratory stock diet. A few protein-deficient rats were included for completeness. After eight weeks on the diets the radioiron-ascorbic acid solution was administered by stomach tube to the three groups of rats.

FIG. 21


RADIOIRON ABSORPTION
% retention of ^{59}Fe
100
80
60
40
20
casein diet
stock diet
Protein-free diet
1
2
3
4
5
6
7
DAYS
EACH RAT GIVEN 0.5 MG. OF IRON + 2 MG. OF ASCORBIC ACID

Results and observations

It is evident from Fig. 21 that the rats on stock diet and those on the casein diet behaved similarly. After a week the rats on stock diet retained an average of 4.6% S.D. ± 1.2 (7 rats). The rats on casein diet retained an average of 3.8% S.D. ± 0.7 (6 rats). These values are close to those obtained in the previous experiment (Fig. 20). The protein-deficient rats, however, retained much more, about 22% but, due to the difficulty of passing the tube safely into the stomach only two rats were tested.

The experiments just described confirm the previous observations that protein-deficient animals have a greater capacity to absorb iron than normal animals, and show that absorption from a standard dose increases with the duration of the deficiency.

IRON STORAGE

EXPERIMENTAL METHODS

The livers and spleens of the rats, previously bled for blood analysis, were removed, weighed and immediately deep-frozen for about an hour. They were then analysed for ferritin and haemosiderin by ion-exchange chromatography on carboxy-methyl-cellulose (Drysdale and Ramsay, to be published). The tissues were chopped and homogenised in nine volumes of ice-cold distilled water. The homogenate was brought to pH 4.9 by the addition of citric acid (0.1M). One ml. of homogenate was pipetted and stirred into the top of a carboxymethyl-cellulose column which had already been washed through by 0.01M citric acid sodium-citrate buffer pH 4.9. About 20 ml. of the same buffer were run through. Ferritin was eluted with 0.025M citrate buffer, pH 5.7 and collected in three

10 ml. volumes. Haemoglobin was removed by running through about 60 ml. of 0.05M citrate buffer + 0.3M sodium chloride, pH. 5.7. Finally haemosiderin was eluted with 0.1N sodium hydroxide and collected in two 10 ml. cuts.

In the original work* ferritin was characterized by crystallization, by electron microscopy and from its solubility and sedimentation properties. Haemosiderin cannot yet be positively identified so satisfactorily, but the haemosiderin fraction was shown not to contain ferritin or haemoglobin, and to account quantitatively for the amount of iron attributable to haemosiderin in other analytical procedures.

*(Drysdale and Ramsay to be published)

Ferritin and haemosiderin iron, collected in pyrex tubes marked at 10 ml., were then estimated by the dipyridyl method. 0.2 ml. of concentrated hydrochloric acid was added to each tube and heated in a boiling water bath. After 5 - 10 min. 1 ml. sodium sulphite, 1.5M and 1 ml. 0.5% 2:2' dipyridyl in 60% redistilled acetic acid were added. The tubes were left in the bath for 1 - 2 hr. The optical density of the pink solution (ferrous dipyridyl) was measured in a Hilger (spekker) absorptiometer, using Ilford filter No. 604 (spectrum green).

EXPERIMENT I IRON STORAGE IN NORMAL RATS

Materials and Method

36 rats weighing between 112 - 182 grams (average 144 grams) were divided randomly into two groups. One group was fed the laboratory stock diet and the other group fed synthetic protein-adequate casein diet "A" (Table I).

After two weeks, three rats from each group were killed weekly for analysis.

Fig 22

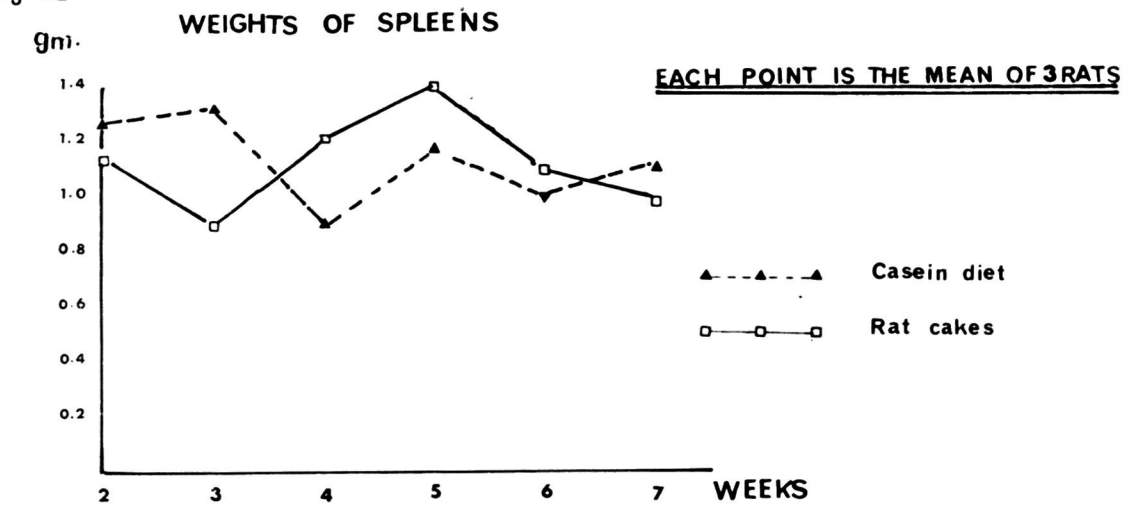
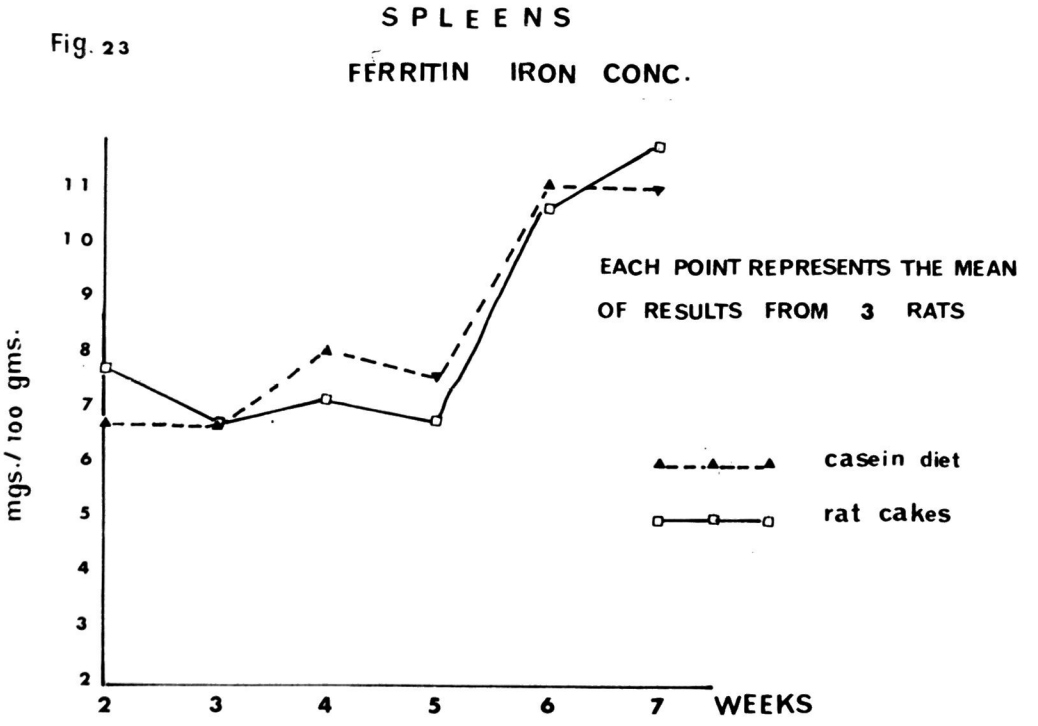

WEIGHTS OF SPLEENS
gm.
EACH POINT IS THE MEAN OF 3 RATS
1.4
1.2
1.0
0.8
0.6
0.4
0.2
2
3
4
5
6
7
WEEKS
Casein diet
Rat cakes

Fig. 23


SPLEENS
FERRITIN IRON CONC.
mgs./100 gms.
11
10
9
8
7
6
5
4
3
2
2
3
4
5
6
7
WEEKS
EACH POINT REPRESENTS THE MEAN
OF RESULTS FROM 3 RATS
casein diet
rat cakes

Results and observations

It was observed that although there was some variation from rat to rat in the same group, the two groups of rats behaved similarly.

Spleen analysis

Weight

There was no significant difference between the weights of the spleens in the two groups (Fig. 22). The average weight of the spleens was 1.13 grams S.D ± 0.2 in the rats on the stock diet and 1.13 grams S.D. ± 0.18 in the rats on the synthetic casein diet. The weights remained constant as the rats gained weight.

Ferritin

The ferritin iron concentration gradually increased in both groups from an initial value of about 7.8 mg./100 grams wet weight to about 11.8 mg./100 grams in the rats on

Fig. 24

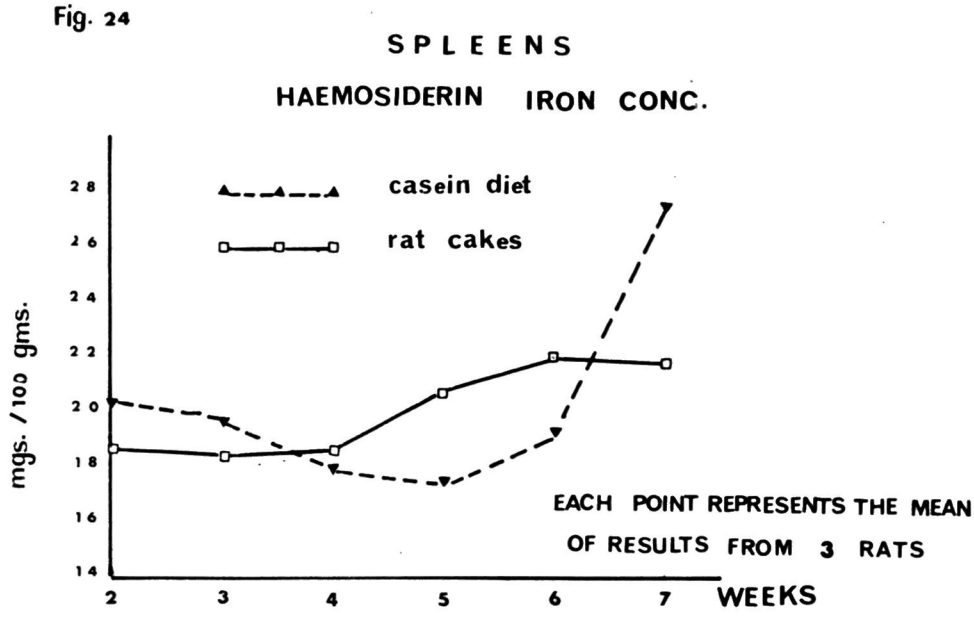
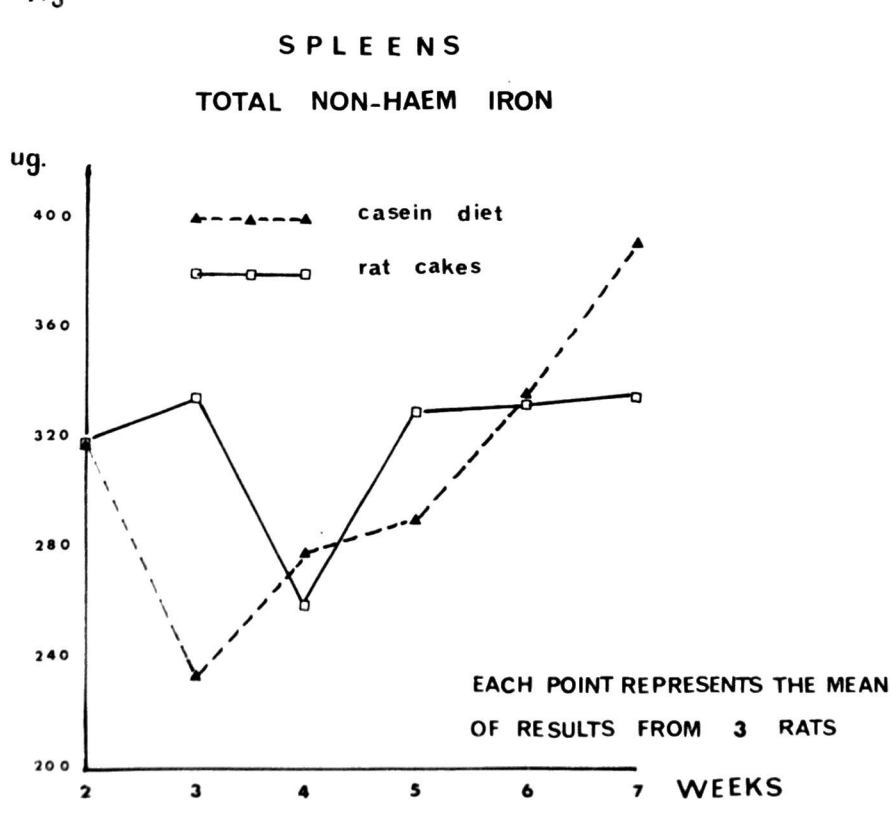

SPLEENS
HAEMOSIDERIN IRON CONC.
casein diet
rat cakes
mgs. / 100 gms.
28
26
24
22
20
18
16
14
EACH POINT REPRESENTS THE MEAN
OF RESULTS FROM 3 RATS
2
3
4
5
6
7
WEEKS

Fig. 25


SPLEENS
TOTAL NON-HAEM IRON
ug.
casein diet
rat cakes
400
360
320
280
240
200
EACH POINT REPRESENTS THE MEAN
OF RESULTS FROM 3 RATS
2
3
4
5
6
7
WEEKS

stock diet in seven weeks, and from 6.8 to 11.1 mg./100 grams in the other group in the same period (Fig. 23). The fact that the average weights of the spleens remained approximately constant throughout the experiments means that more ferritin had been deposited in the spleens.

Haemosiderin

Haemosiderin iron concentration showed the same tendency to increase gradually in the rats on stock diet, rising from 18.6 to 21.8 mg./100 grams. In the casein diet group, though there was fluctuation from week to week, the tendency to increase is apparent especially in the last two weeks when it rose to 27.5 mg./100 gram (Fig. 24). This would also mean that the haemosiderin content of the spleen in both groups gradually increased.

The total non-haem iron (ferritin + haemosiderin) in the spleens (Fig. 25) showed the same pattern of gradual increase in both groups but more so in the casein diet group. There was, however, considerable fluctuation from week to week.

fig 26

SPLEENS

PERCENTAGE $\frac{\text{HAEMOSIDERIN}}{\text{TOTAL NON-HAEM IRON}}$

EACH POINT IS A MEAN OF 3 RATS

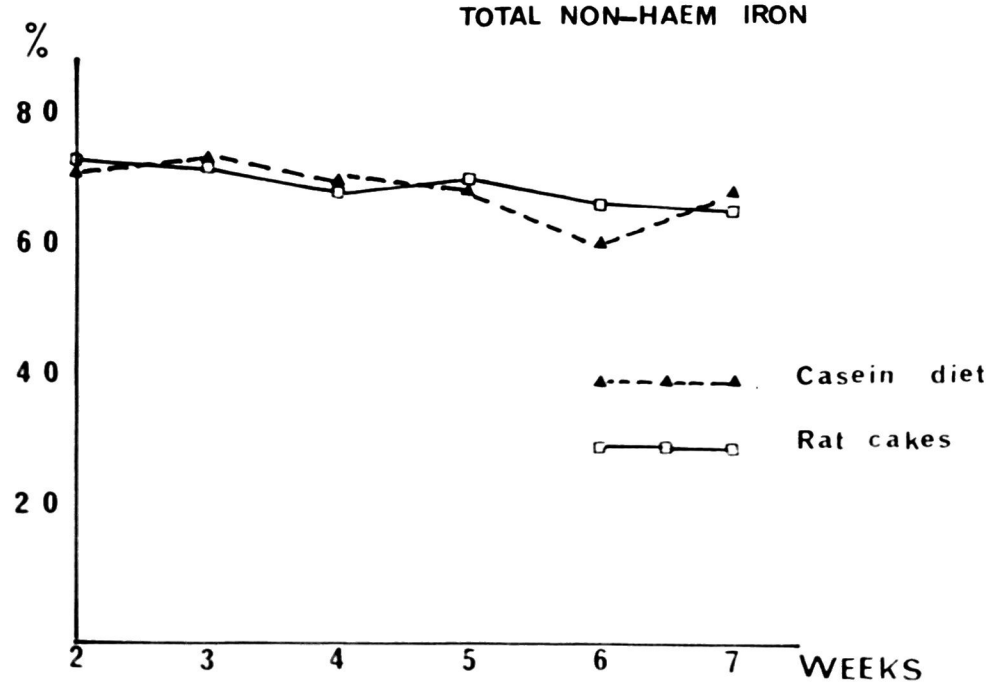


Fig 27

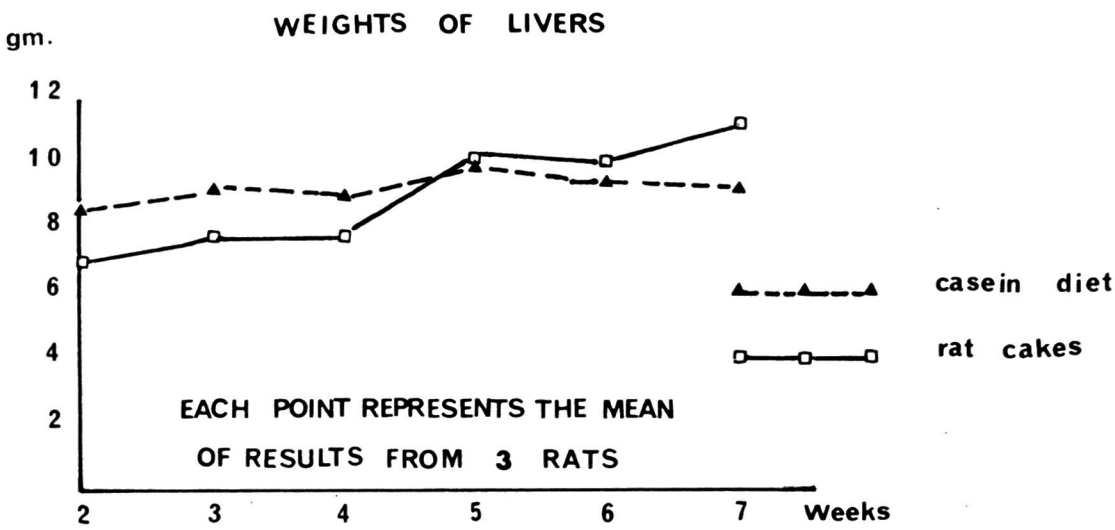
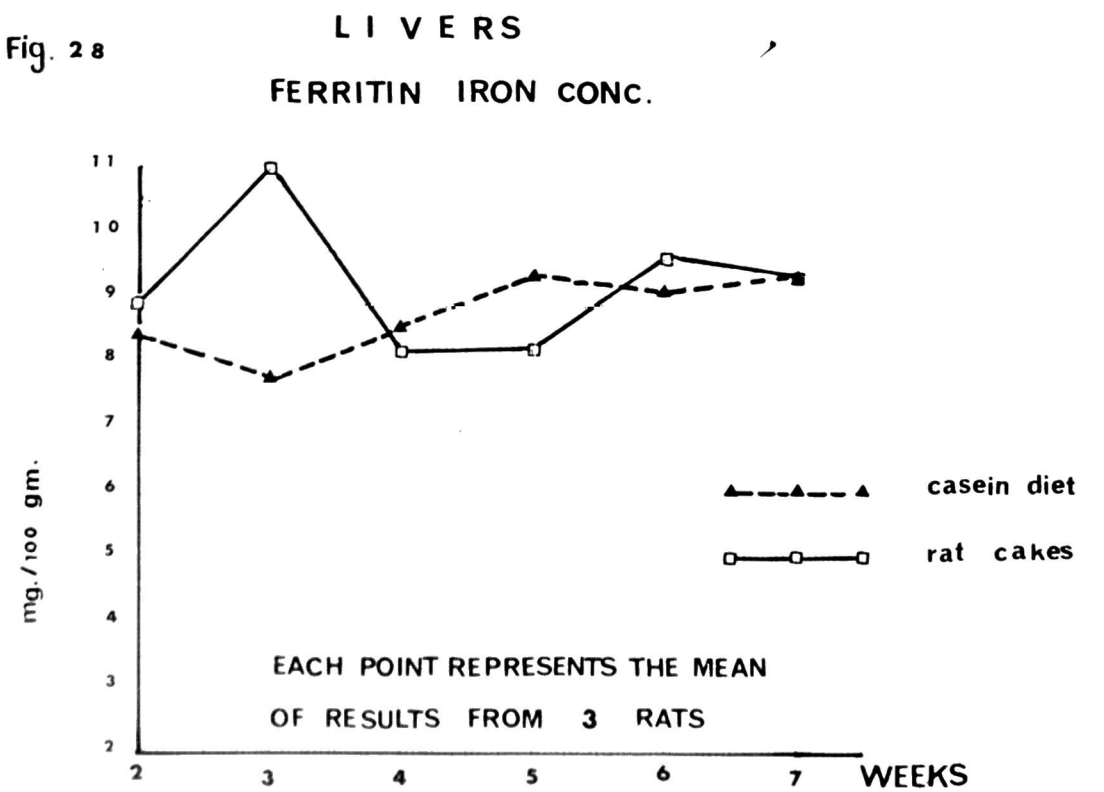

WEIGHTS OF LIVERS
gm.
12
10
8
6
4
2
EACH POINT REPRESENTS THE MEAN
OF RESULTS FROM 3 RATS
2
3
4
5
6
7
Weeks
casein diet
rat cakes

Fig. 28


LIVERS
FERRITIN IRON CONC.
mg./100 gm.
11
10
9
8
7
6
5
4
3
2
EACH POINT REPRESENTS THE MEAN
OF RESULTS FROM 3 RATS
2
3
4
5
6
7
WEEKS
casein diet
rat cakes

It can be seen from Fig. 26 that the haemosiderin iron expressed as a percentage of the total non-haem iron varied from 62 - 75% with an average of 70% in both groups. The ratio of haemosiderin to ferritin was about 2.4:1.

Liver analysis

Weights

Fig. 27 shows that liver weights of the rats on stock diet gradually increased from 7 to 11 grams, while in the rats on casein diet it rose from 8.6 to 9.2 grams in seven weeks. The average liver weights throughout the experiment were 8.9 grams S.D. $\pm$ 1.6 for rats on stock diet and 9.2 grams S.D. $\pm$ 1.05 for those on casein diet. This difference of 0.3 grams is obviously insignificant.

Ferritin

The levels of ferritin iron concentration in both groups agreed reasonably well, with some fluctuation about the mean. In contrast to the observation in the spleens,

Fig. 29

LIVERS

HAEMOSIDERIN IRON CONC.

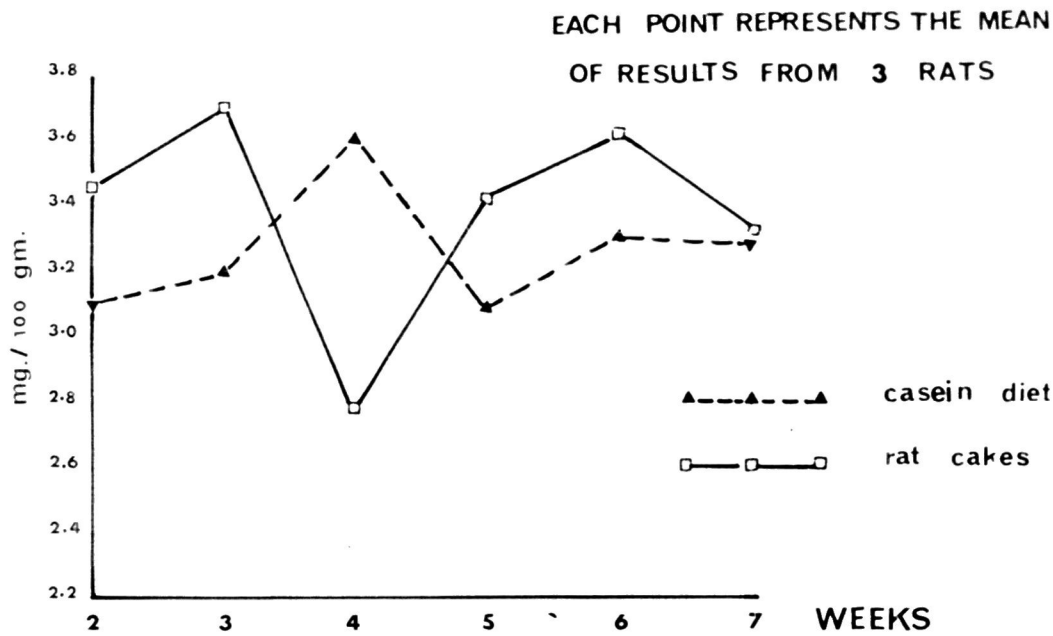


Fig 30

LIVERS

TOTAL NON HAEM IRON

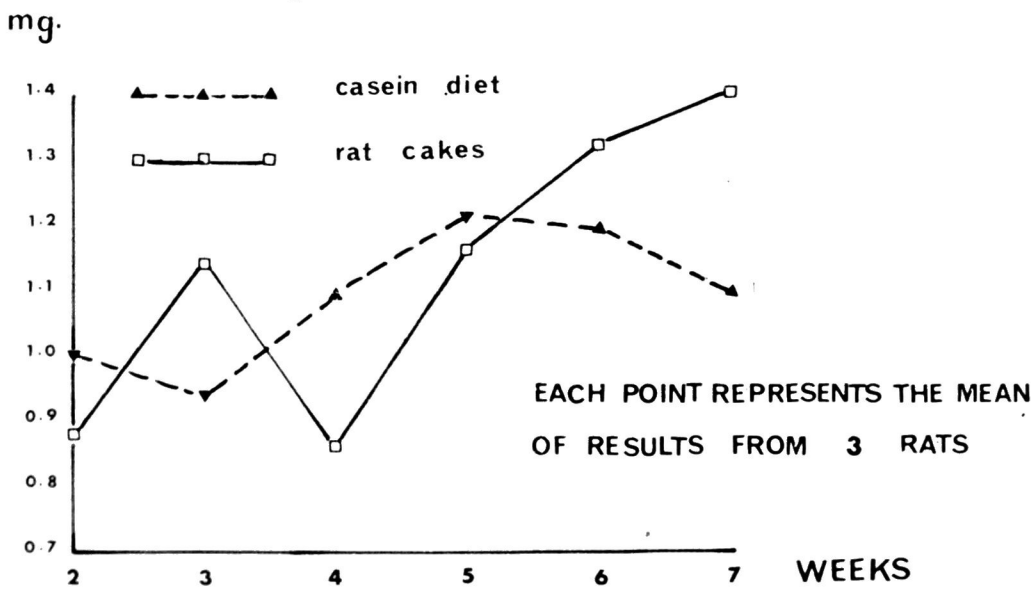
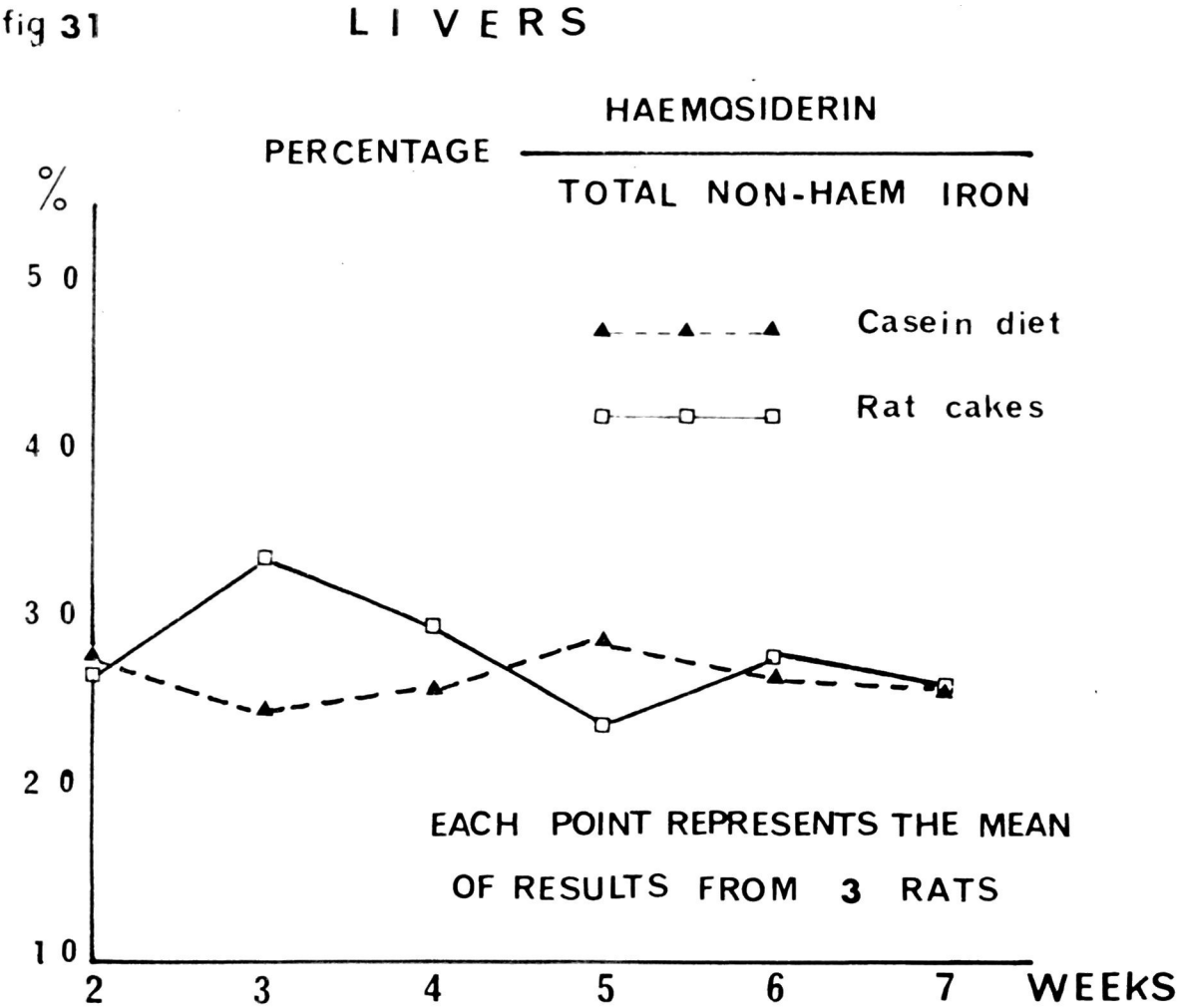


fig 31


LIVERS
HAEMOSIDERIN
PERCENTAGE
TOTAL NON-HAEM IRON
%
50
40
30
20
10
Casein diet
Rat cakes
EACH POINT REPRESENTS THE MEAN
OF RESULTS FROM 3 RATS
2
3
4
5
6
7
WEEKS

liver ferritin remained approximately constant, with no tendency to increase. The initial concentrations for the two groups were 8.5 and 8.9 mg./100 grams and the final concentration was 9.3 mg/100 grams for both groups (Fig. 28).

Haemosiderin

The haemosiderin iron concentration in the two groups also behaved similarly throughout the experiment ranging between 2.8 - 3.7 mg./100 grams in the rats on stock diet and between 3.1 - 3.6 mg./100 grams in the other group (Fig. 29). However, due to the slight increase in the liver weights, there was also a rise in the total non-haem iron (Fig. 30).

In the livers as distinct from the spleens, haemosiderin iron accounted for only about 25% of the total non-haem iron (Fig. 31) and the ratio of haemosiderin to ferritin was about 1:3.

EXPERIMENT II IRON STORAGE IN PROTEIN-DEFICIENT RATS (A)

Materials and Method

Young rats weighing between 87 - 197 grams (average 149 grams) were divided at random into three groups of 18, A, B and C. Group A was fed the diet in which protein was provided as casein; group B received an equicaloric diet in which the casein was replaced by zein which is defective in lysine and tryptophane. Group C was given the protein-free diet (see Tables 1, 2 and 3).

After an initial period of three weeks, two rats from each group were killed weekly for analysis.

Fig. 32 AVERAGE WEIGHTS OF SPLEENS

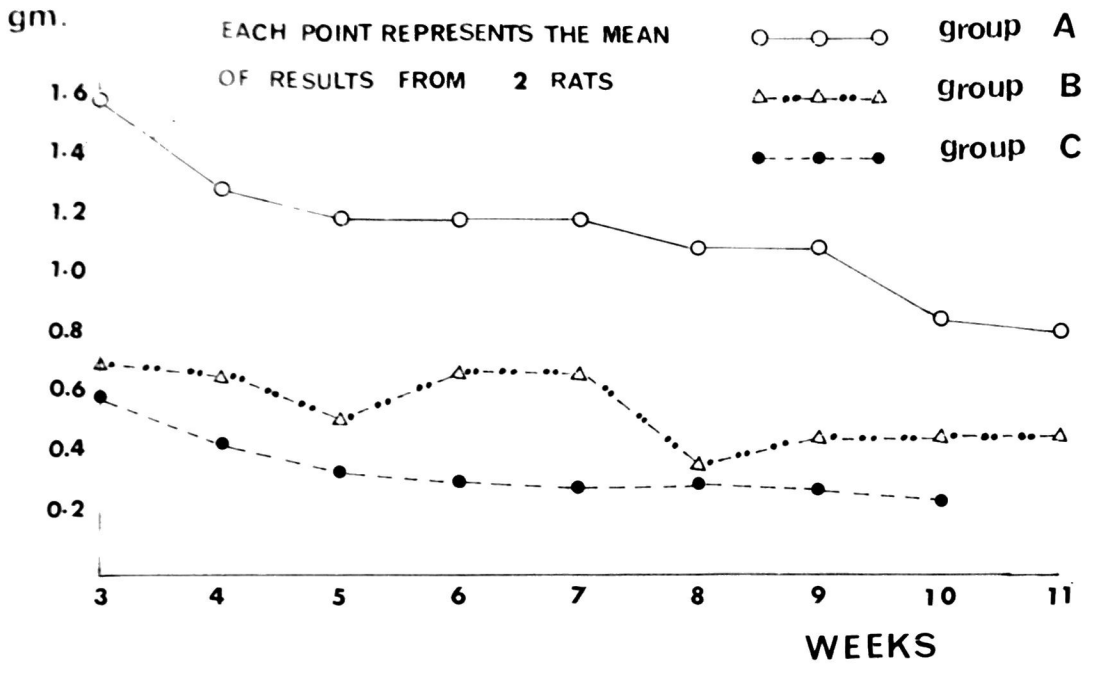
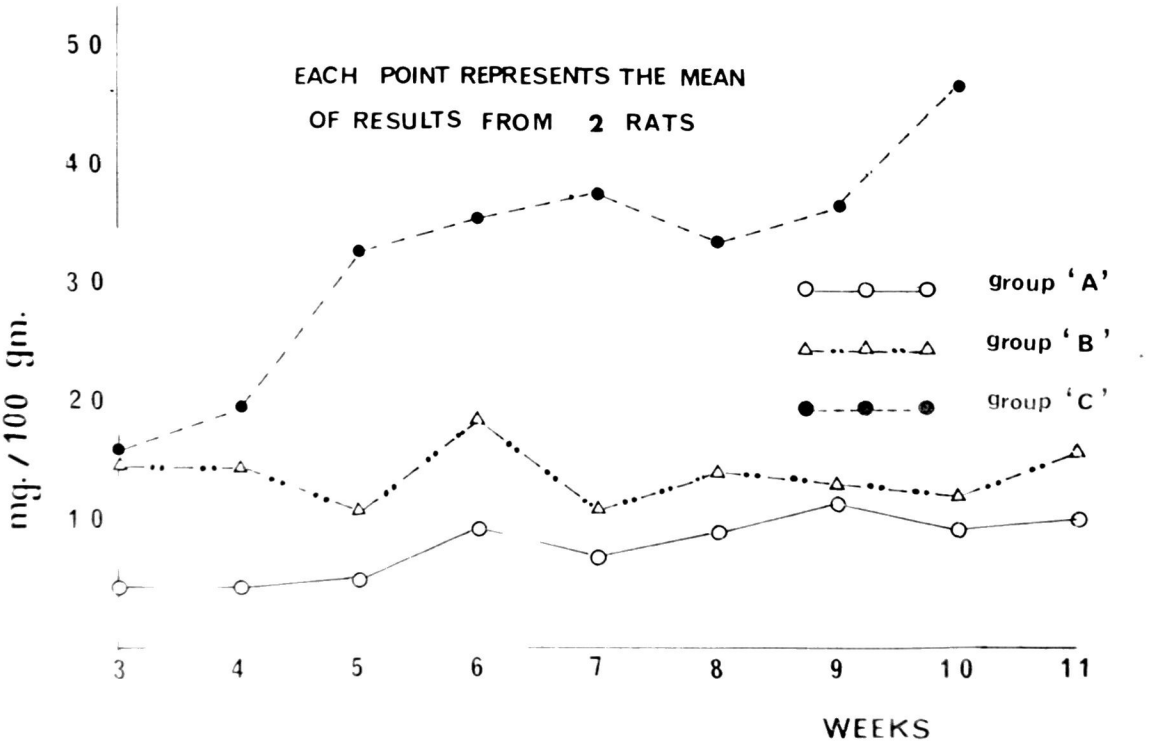


Fig. 33 SPLEEN

Ferritin Iron Conc.



Results and observations

Spleen Analysis

Weight

There was considerable diminution in the weights of the spleens of the rats in group B and C to about 0.46 grams and 0.25 grams respectively after ten weeks. In group A the weights maintained an average level of about 1.1 grams (Fig. 32) which agrees with the finding in normal rats in the previous experiment. It was observed, however, that the weights showed a drop to 0.82 grams in the last two weeks. There was no obvious explanation for this discrepancy. A bigger number of rats would need to be analysed to evaluate its significance.

fig. 34

SPLEEN

Haemosiderin Iron Conc.

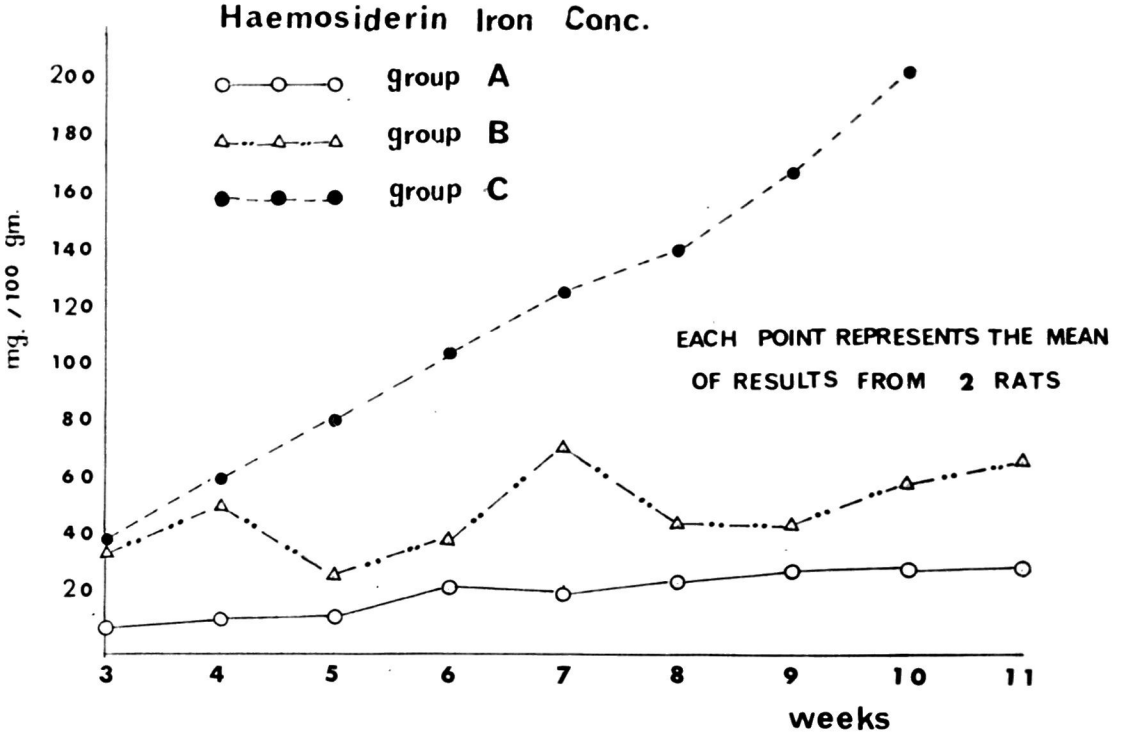
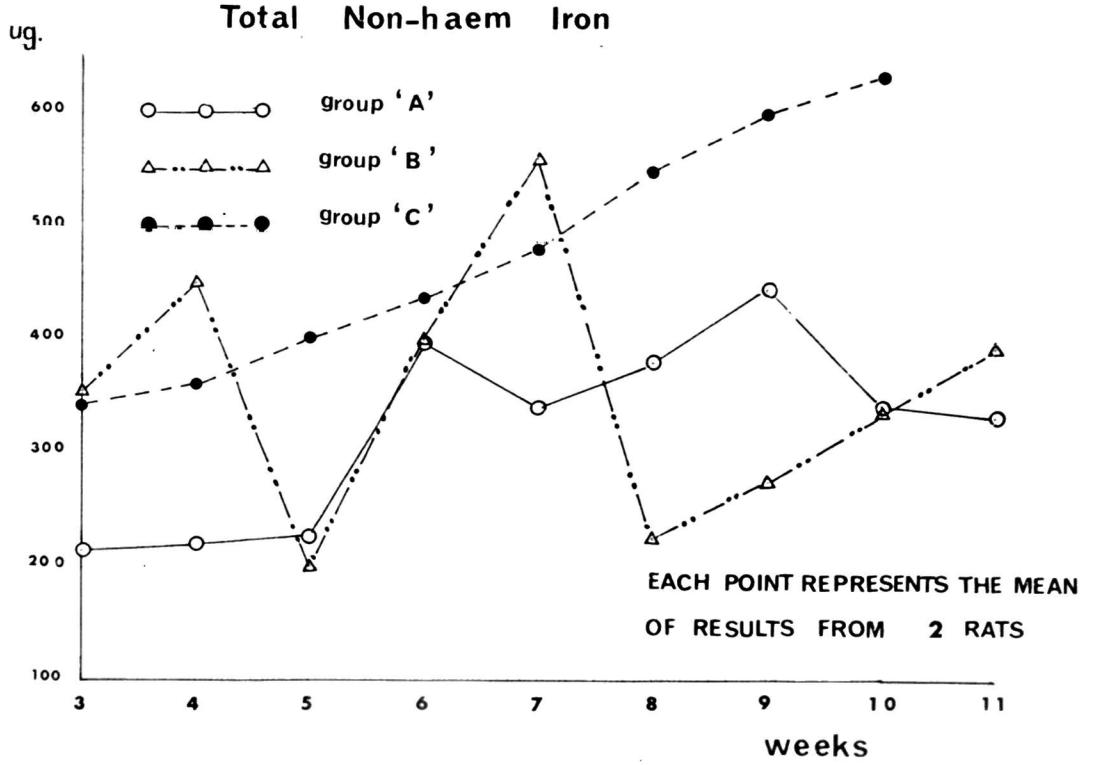


fig. 35

SPLEEN

Total Non-haem Iron

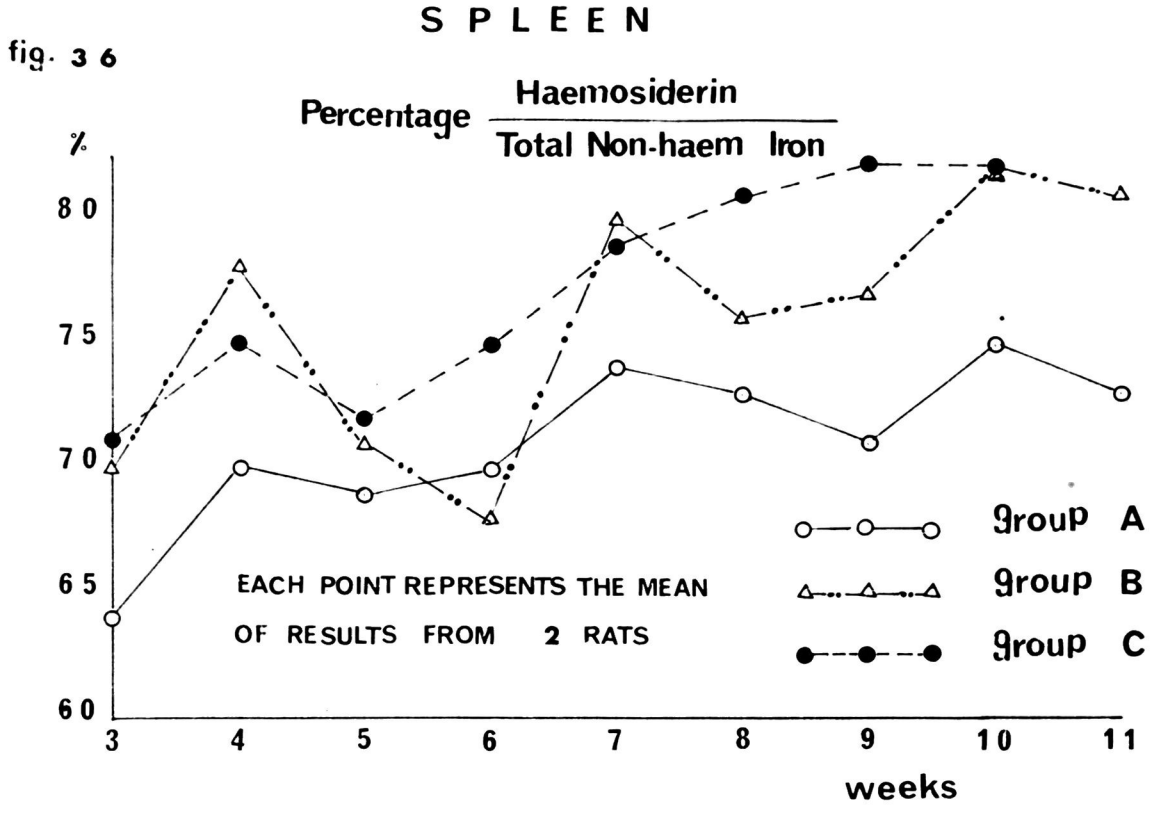


It is interesting to note that the maximum loss in the weights of the spleens in groups B and C took place in the first three weeks. After five weeks there was practically no change. It would be desirable to find out when the effect of protein deficiency started to appear.

Ferritin

The ferritin iron concentration in group C rose from 16 mg./100 gram after three weeks to about 47 mg./100 grams after ten weeks. In group B it rose from 15 to 17 mg./100 grams (Fig. 33). This rise in ferritin iron concentration in the spleens of the normal rats (group A) does not mean that the ferritin content had been doubled, because of the observed discrepancy in the weights of the spleens in the last two weeks. It is shown in Figs. 32 and 33 that when the ferritin iron concentration was about 5 mg./100 grams, the average weight of the spleens was 1.6 grams, while it was only 0.82 grams when the ferritin iron concentration became 10.5 mg./100 grams.

fig. 36


SPLEEN
Percentage Haemosiderin / Total Non-haem Iron
%
80
75
70
65
60
3
4
5
6
7
8
9
10
11
weeks
EACH POINT REPRESENTS THE MEAN
OF RESULTS FROM 2 RATS
Group A
Group B
Group C

At three weeks the ferritin iron concentrations in the spleens of group B and C were already three times as much as that of group A, and continued to increase in group C up to 10 weeks, while in group B it remained at an approximately constant level. At ten weeks the ferritin iron concentration in group C was about five times that of group A.

Haemosiderin

The haemosiderin iron concentration showed the same pattern as ferritin but at a higher level (Fig. 34). At three weeks the average concentration was 10 mg./100 grams in A, 36 mg./100 grams in B and 40 mg./100 grams in C - four times as much as in A. There was gradual increase in A, rising to 30 mg./100 grams in eleven weeks. In group B it fluctuated a great deal, rising to 68 mg./100 grams in eleven weeks. In group C, however, there

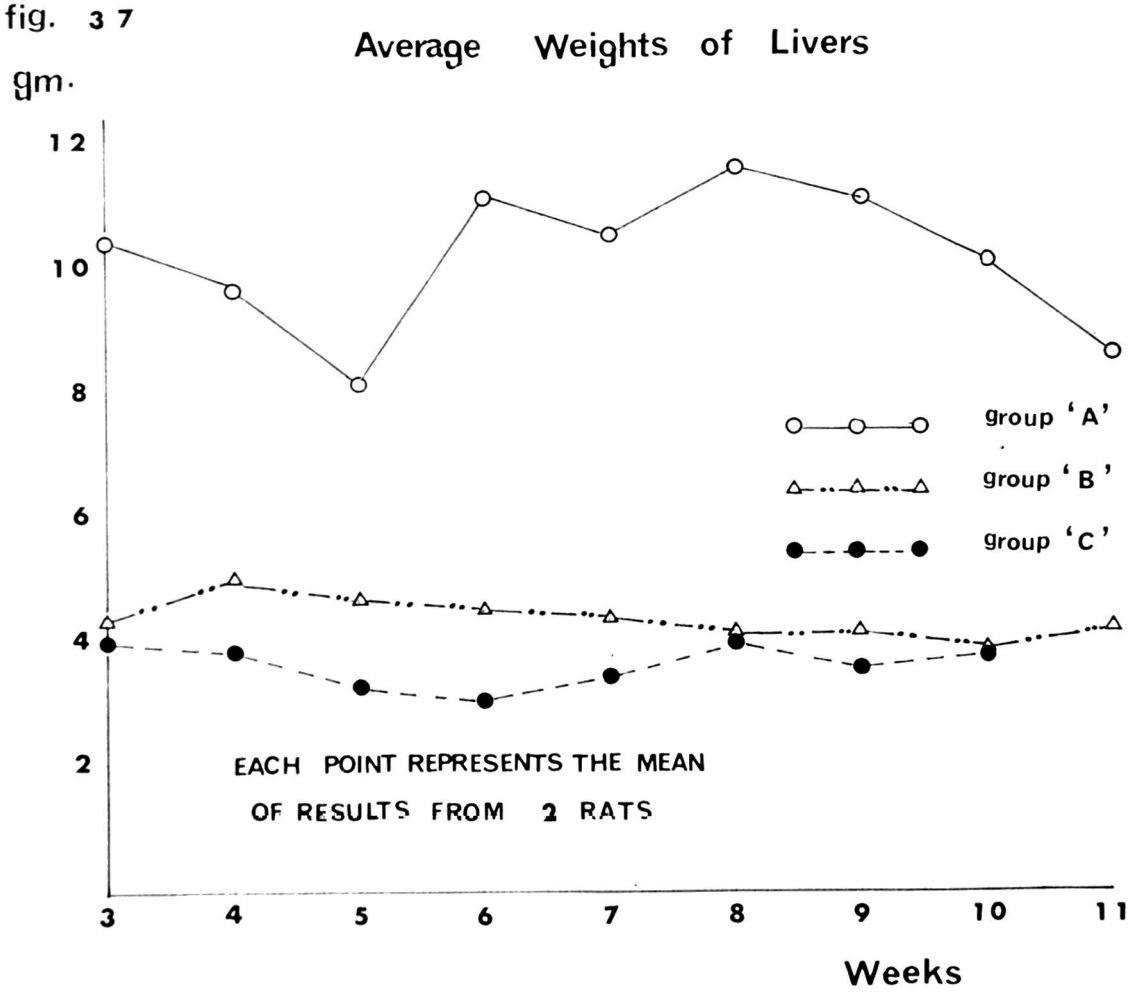
was a striking continuous rise up to 205 mg./100 grams in ten weeks, about seven times as much as in group A and about three times as much as in group B.

This increase in the haemosiderin concentration in the spleens of groups B and C started in the first three weeks, and continued throughout the experiment, being more marked in group C.

As the ferritin and haemosiderin iron concentrations increased in group C, so did the total non-haem iron stored (Fig. 35). In the spleens of groups A and B there was considerable fluctuation, so that it is difficult to say whether the content of stored iron in these spleens changed significantly. More rats would need to be analysed to clarify this point. The percentage of total non-haem iron stored as haemosiderin went up to 82% in group B and C and up to 75% in A (Fig. 36). There was considerable fluctuation in

in group B. The average percentage throughout the experiment for group A was 71% S.E. ± 1.25 and for C it was 78% S.E. 1.59, a difference of 7% with standard error of ± 2 is very significant. At the end of the experiment the ratio of haemosiderin to ferritin iron in group C,B, and A was 5:1, 4:1 and 3:1 respectively.

fig. 37


Average Weights of Livers
gm.
12
10
8
6
4
2
group 'A'
group 'B'
group 'C'
EACH POINT REPRESENTS THE MEAN
OF RESULTS FROM 2 RATS
3
4
5
6
7
8
9
10
11
Weeks

Liver analysis

Weight

In group A the liver weight was 10.5 grams at three weeks and remained about that level throughout the experiment with some fluctuations. In groups B and C, however, the liver weights at three weeks were 4.4 and 4 grams respectively, and remained at these levels without any significant change (Fig. 37). In both cases the loss in liver weights occurred in the first three weeks.

Ferritin

The ferritin iron concentration rose from about 15 mg./100 grams in three weeks to about 30 mg./100 grams in ten weeks in group C, while in group B it rose from about 15 to 20 mg./100 grams. In group A, however, it remained at a low level varying between 6 and 8 mg./100 g. (Fig. 38).

fig. 38

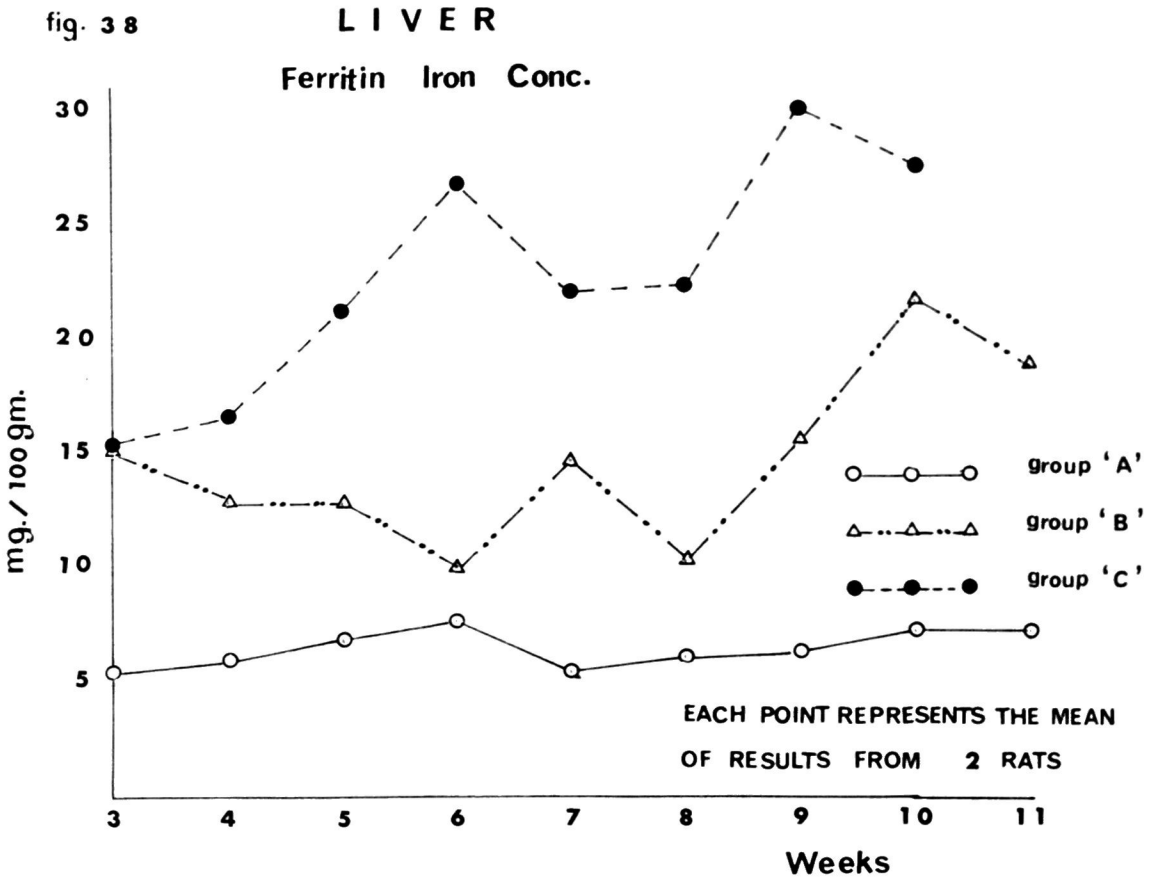

LIVER
Ferritin Iron Conc.
mg./100 gm.
30
25
20
15
10
5
group 'A'
group 'B'
group 'C'
EACH POINT REPRESENTS THE MEAN
OF RESULTS FROM 2 RATS
3
4
5
6
7
8
9
10
11
Weeks

fig. 39

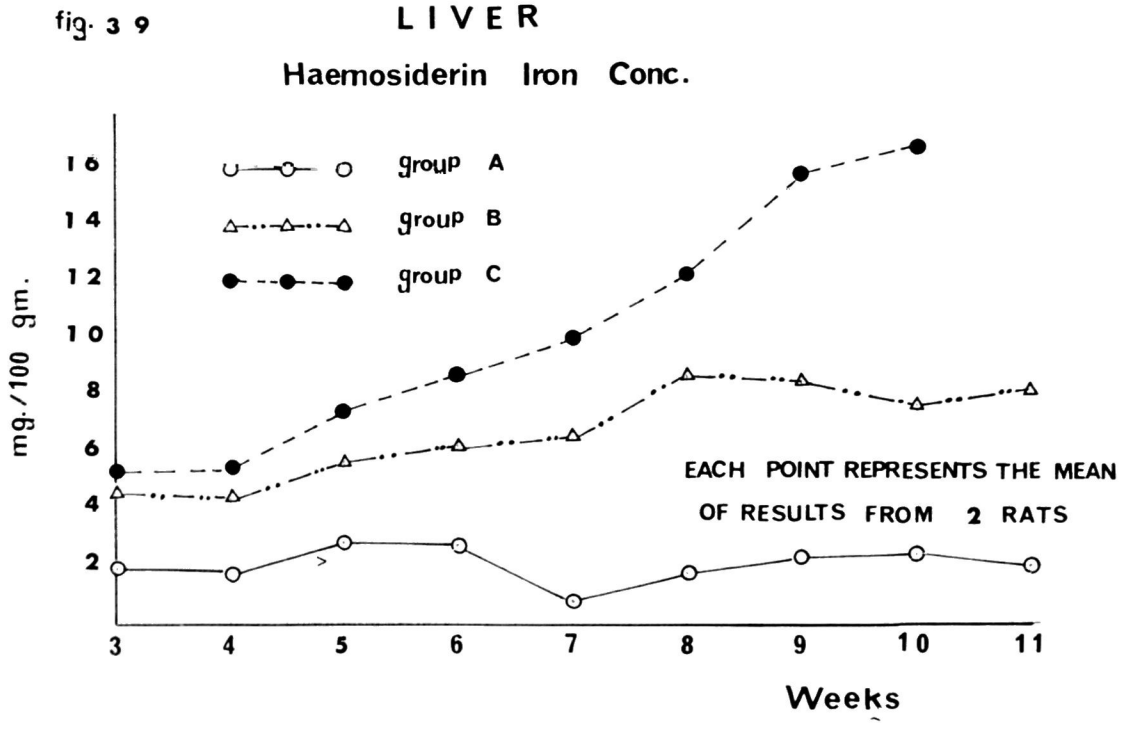

LIVER
Haemosiderin Iron Conc.
mg./100 gm.
16
14
12
10
8
6
4
2
group A
group B
group C
EACH POINT REPRESENTS THE MEAN
OF RESULTS FROM 2 RATS
3
4
5
6
7
8
9
10
11
Weeks

Fig. 40

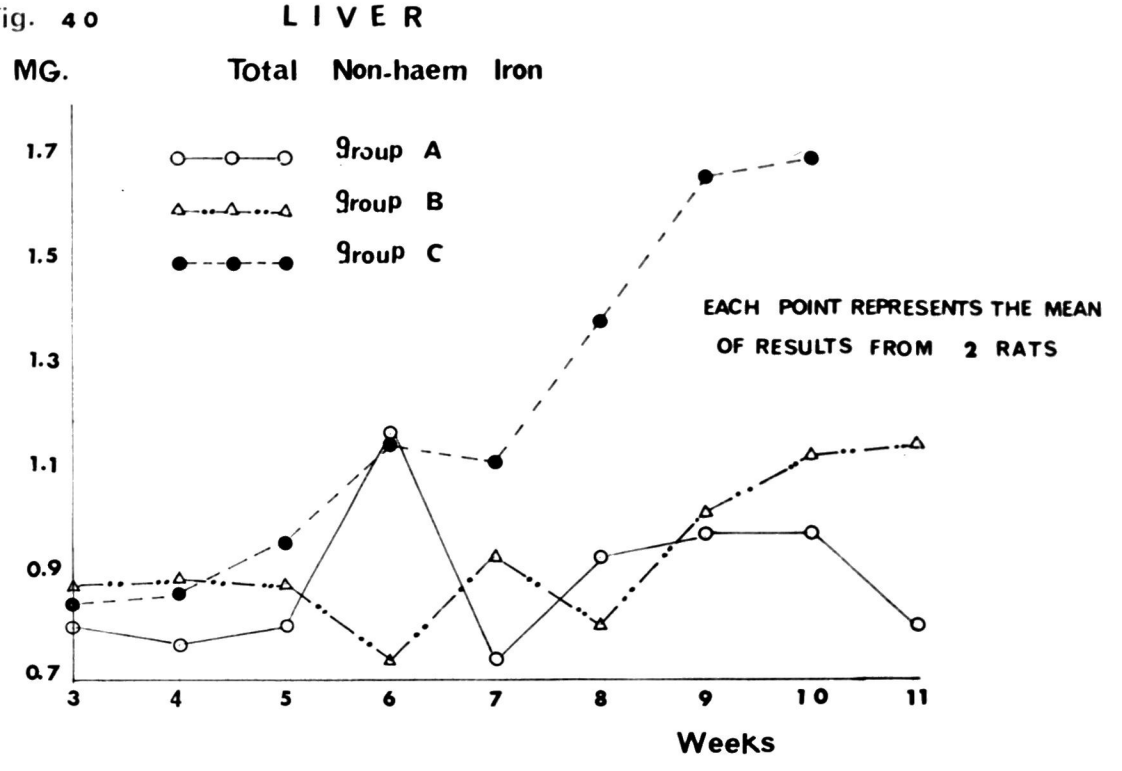
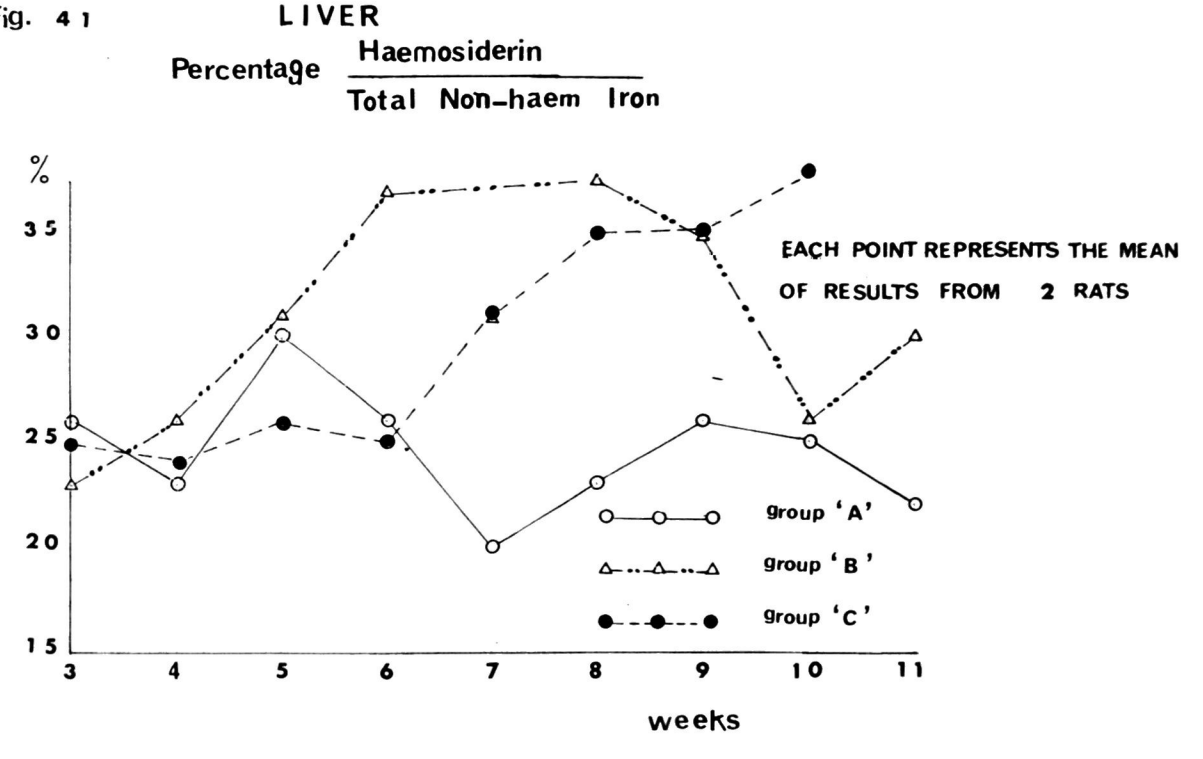

LIVER
MG.
Total Non-haem Iron
1.7
1.5
1.3
1.1
0.9
0.7
Group A
Group B
Group C
EACH POINT REPRESENTS THE MEAN
OF RESULTS FROM 2 RATS
3
4
5
6
7
8
9
10
11
Weeks

Fig. 41


LIVER
Percentage Haemosiderin / Total Non-haem Iron
%
35
30
25
20
15
EACH POINT REPRESENTS THE MEAN
OF RESULTS FROM 2 RATS
group 'A'
group 'B'
group 'C'
3
4
5
6
7
8
9
10
11
weeks

Haemosiderin

Fig. 39 shows that the haemosiderin iron concentration increased from 5 mg./100 grams to about 17 mg./100 grams in group C and from 5 to 8 mg./100 grams in group B, while in A it stayed at the low normal range of 2 - 3 mg. /100 gram.

The total non-haem iron showed a similar increase in group C, rising to 1.7 mg. There was considerable fluctuation in group A and B, but in group B there was a slight gradual increase in the last three weeks, up to 1.15 mg. In the normal rats however it remained below 1.0 mg. except in the sixth week when it went up to 1.17 mg. (Fig. 40).

The percentage of non-haem iron present as haemosiderin rose to 38% in group C but remained at about 25% in group A while in group B there was marked variation from week to week, falling to 26% and rising to 37% (Fig. 41). The striking difference between A and C started to appear only after the sixth week. The number of rats analysed after the sixth week was too small to allow for statistical analysis of this apparent difference.

The ratio of haemosiderin iron to ferritin iron was 1:3 in the control group, while in group B and C it rose to 2:5 and 3:5 respectively.

This experiment showed that rats on a diet totally deficient in protein store significantly more iron than normal rats in both liver and spleen. Much of the excess iron is found as haemosiderin, but there is a considerable increase also in ferritin iron. When the diet contained the poor quality protein zein the results were intermediate between those in the normal and completely deficient animals. It was decided therefore not to continue with the zein diet, but to repeat the experiment with more rats and with larger rats, to test whether their relatively diminished iron requirements would influence the results. It seemed also that it would be of interest to commence observations earlier than three weeks after the animals were placed on the experimental diets.

EXPERIMENT III. IRON STORAGE IN PROTEIN DEFICIENT RATS (B)

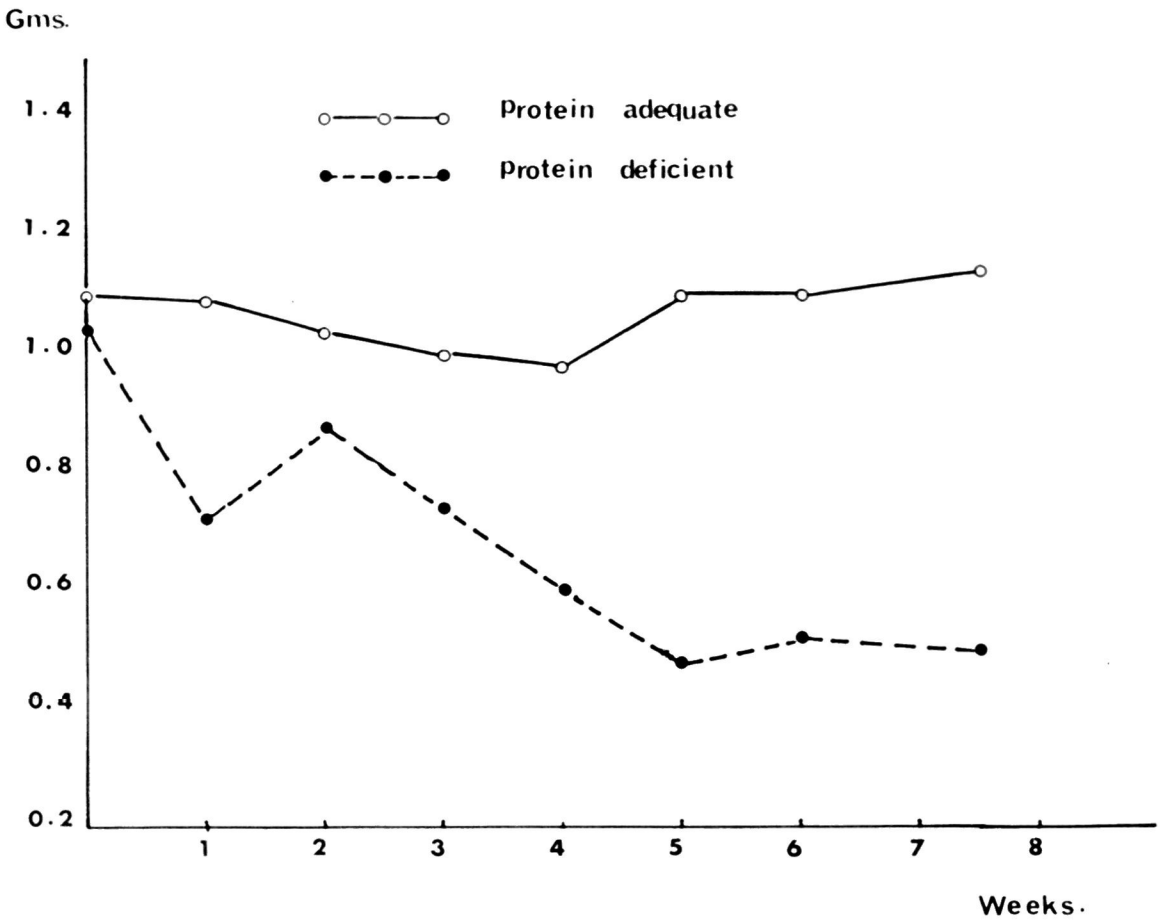
Materials and Method

Rats weighing between 250 and 360 grams (average 305 grams) were divided into two groups of 48. One group was fed the casein diet "A" and the other the protein-free diet "C".

Six rats from each group were killed weekly for analysis, commencing the day after the experiment was begun.

FIG. 42

Average Weights of Spleens



☆ Each Point is an average of 6 rats.

Results and observations

Spleen analysis

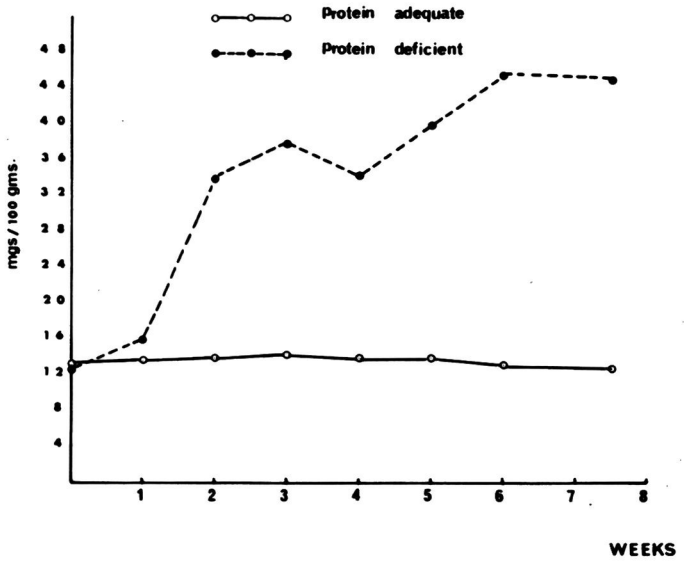
Weight

The spleens of the rats on the casein diet maintained an essentially constant weight throughout the experiment with an average of 1.06 grams S.D. ± 0.15. The discrepancy observed in the weights of spleens in the comparable group of rats in the previous experiment was not seen in this experiment. In the protein-deficient rats the spleen weights again showed the gradual decline up to the fifth week, with no further loss in the sixth and seventh week. It was observed, however, that the decline in weight was less striking than in the previous experiment when younger rats were used. In seven weeks the weights declined to 0.5 grams and 0.3 grams in this and the previous experiment respectively (Fig. 42).

Fig. 43

SPLEENS

Average Ferritin Iron Conc.

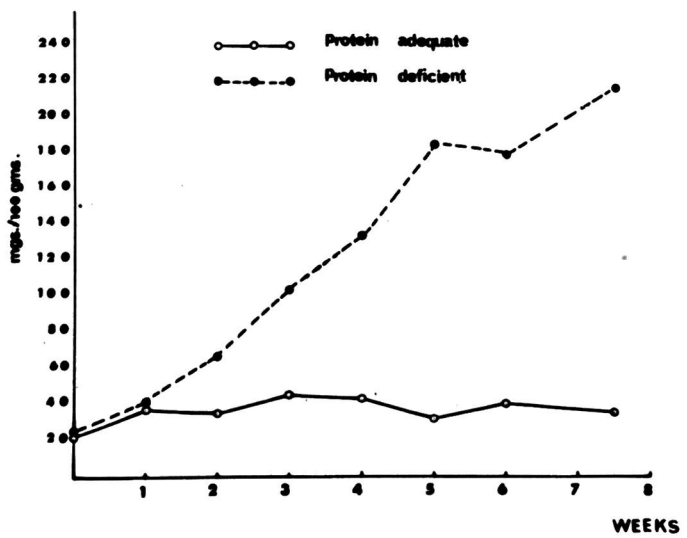


☆ Each Point is an average of 6 rats

Fig 44

SPLEENS

Average Haemosiderin Iron Conc.



☆ Each Point is an average of 6 rats.

Ferritin

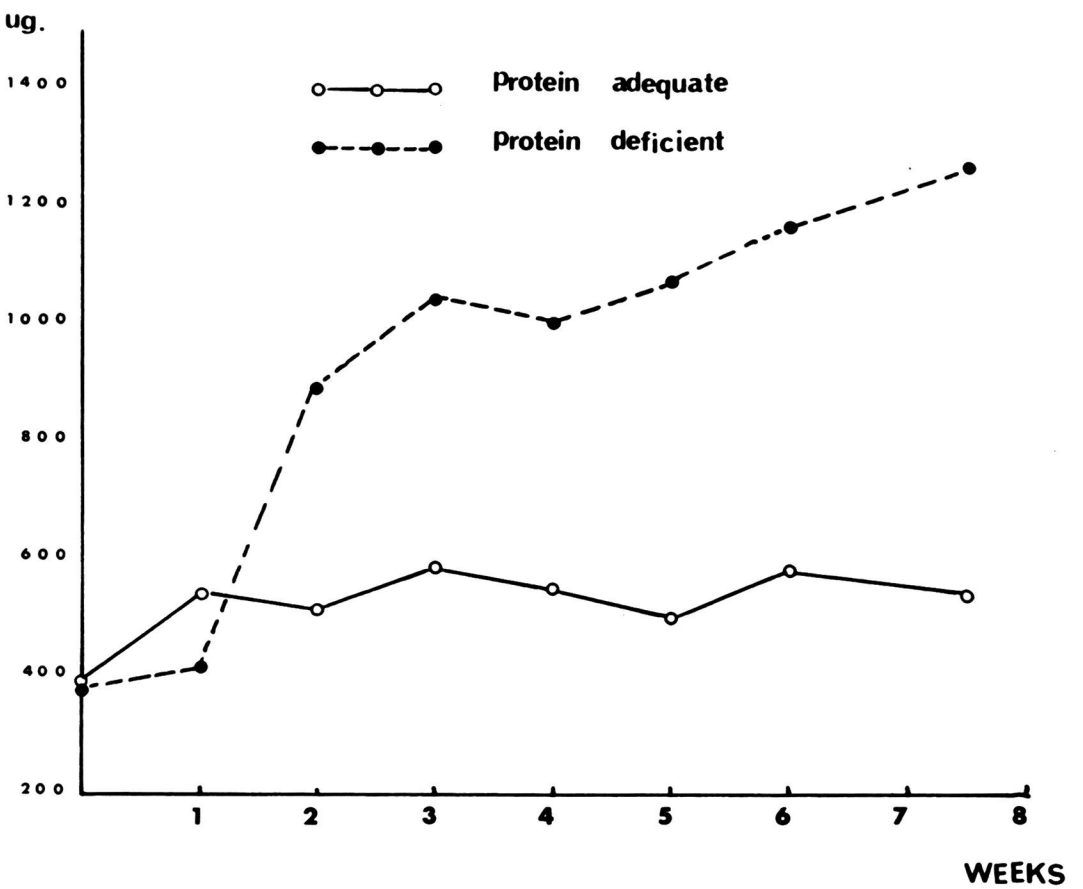
Fig. 43shows that ferritin iron concentration of the normal rats was initially higher than in the smaller rats used in the previous experiments (Fig. 23, 33 and 43). It started at 13 mg./100 grams and maintained this level throughout the experiment, while in the previous two experiments it started at a level around 6 - 7 mg./100 grams and gradually increased as the rats grew bigger to reach a comparable level after about seven to nine weeks.

In the protein deficient rats, the initial ferritin iron concentration was also about 13 mg./100 grams and gradually increased to a level of about 46 mg./100 grams after seven weeks, more than three-fold increase. The total amount of ferritin rose from about 140 μg. to about 230 μg. in the protein-deficient rats in spite of the decline in the weights of spleens. In the normal rats there was no significant change from the initial value of about 140 μg.

Fig. 45

SPLEENS

Total Non-hæm Iron



☆ Each point is an average of 6 rats

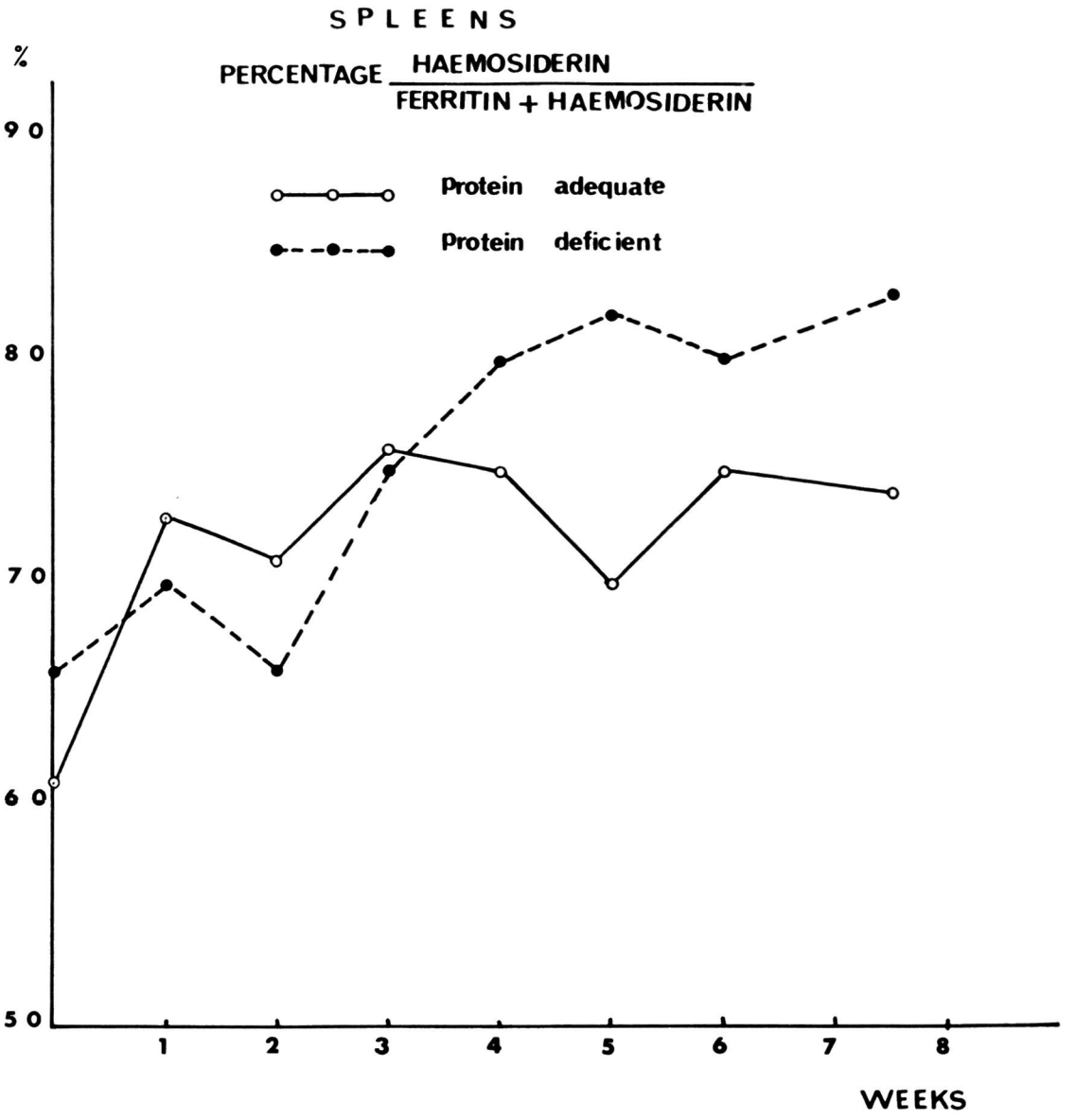
Haemosiderin

The haemosiderin iron concentration, however, showed a very striking difference. In both groups it started at the same level of about 25 mg./100 grams and remained varying between 25 - 40 mg./100 grams in the normal rats, while it progressively increased in the protein-deficient to about 220 mg./100 grams, after seven weeks, approximately nine-fold increase. This means more than four times increase in the haemosiderin content of the spleens of the protein-deficient rats.

It is interesting to note that the change in ferritin and haemosiderin iron concentrations started to appear immediately after the first week, (Fig. 44).

The total non-haem iron in the spleens of the normal rats varied between 400 - 590 μg. in the course of the experiment, while in the protein-deficient rats it gradually rose to 1270 μg., about three-fold increase, (Fig. 45).

Fig. 46

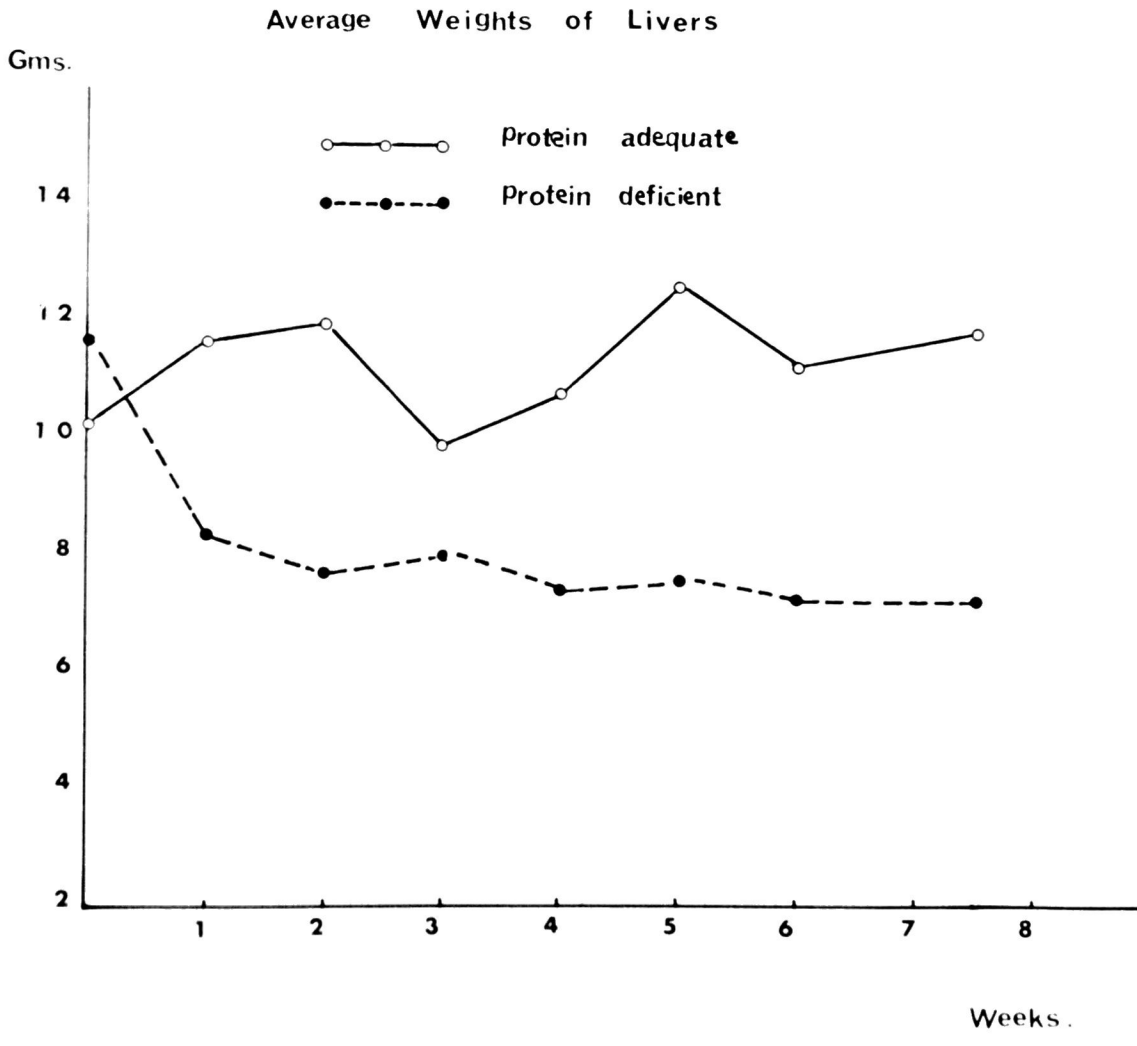


☆ Each point is an average of 6 rats

The percentage of this total non-haem iron present as haemosiderin iron rose to about 75% in the third week in both groups, and remained at or below this level in the normal animals while in the protein-deficient it gradually rose to 83% (Fig. 46). The average percentage of haemosiderin in all the normal rats after the third week was 73% (S.E. ± 1.15). In the protein-deficient it was 81% (S.E. ± 0.84). The difference of 8% with standard error of ± 1.4 is evidently highly significant.

The ratio of haemosiderin iron to ferritin iron was initially 2:1 in both groups. At the end of the experiment it was 2.8:1 in the normal and 4.7:1 in the protein-deficient rats.

FIG. 47



☆ Each point is an average of 6 rats

Liver analysis

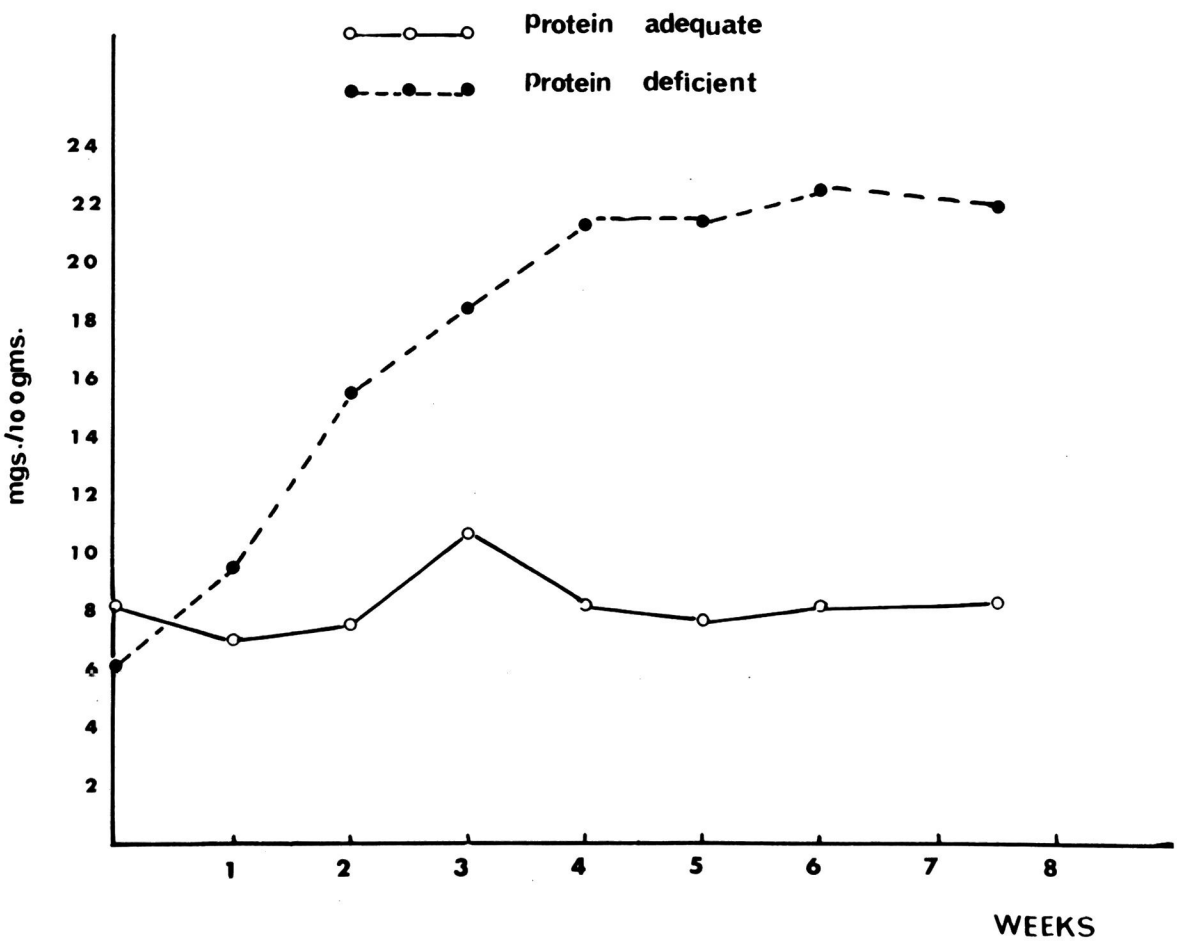
The decline in the weights of the livers of protein-deficient rats observed in the previous experiment (Fig. 37) also occurred in this experiment (Fig. 47), although it was not so striking. The liver weight dropped to 7.2 grams in seven weeks, while in the previous experiment, with younger rats, it dropped to 4.0 grams in the same period. The maximum loss in the weights occurred in the first week, with very slight loss up to the fourth week, and none afterwards. In the normal rats, the weights fluctuated between 10.3 to 12.5 grams throughout the experiment (average 11.1 grams S.D. $\pm$ 1.7).

It appears, thenthat the younger the rats, the more they are prone to loss in tissue weight when protein is withdrawn from the diet.

Fig. 48

LIVERS

FERRITIN IRON CONC.



☆ Each Point is an average of 6 rats.

Ferritin

In the normal rats the ferritin iron concentration remained at a constant level of about 8 mg./100 grams throughout the experiment with the usual slight fluctuations (Fig. 48). In the protein-deficient rats, however, it rose from about 7 to about 22 mg./100 grams in seven weeks - a three-fold increase. Again more ferritin was deposited in the liver in spite of the loss in weight.

Haemosiderin

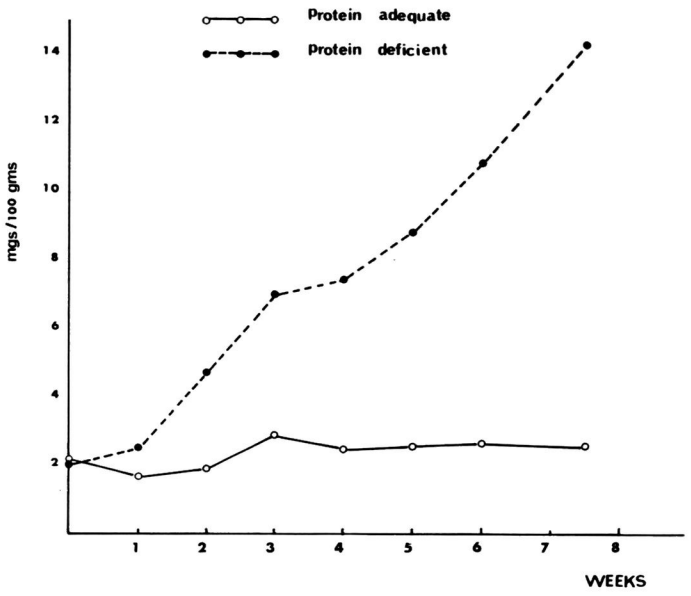
Fig. 49 shows that haemosiderin iron concentration in the livers of normal rats did not vary from the normal range of 2 - 3 mg./100 grams as distinct from the protein-deficient rats which showed a significant rise to about 14 mg./100 grams (six-fold increase) in seven weeks, indicating that a greater proportion of the excess iron was deposited as haemosiderin.

In the liver as well as the spleen, the ferritin and haemosiderin concentrations started to increase immediately after the first week in the protein-deficient rats.

Fig. 49

LIVERS

HAEMOSIDERIN IRON CONC.

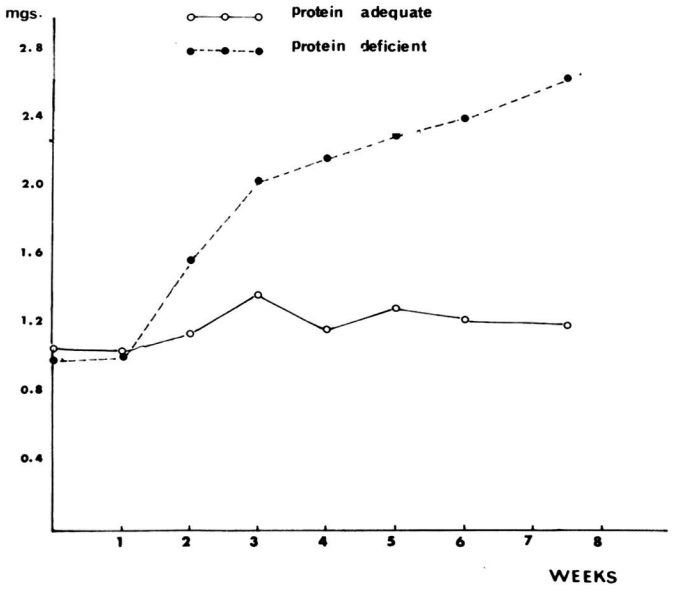


☆ Each point is an average of 6 rats

Fig. 50

LIVERS

TOTAL NON-HAEM IRON

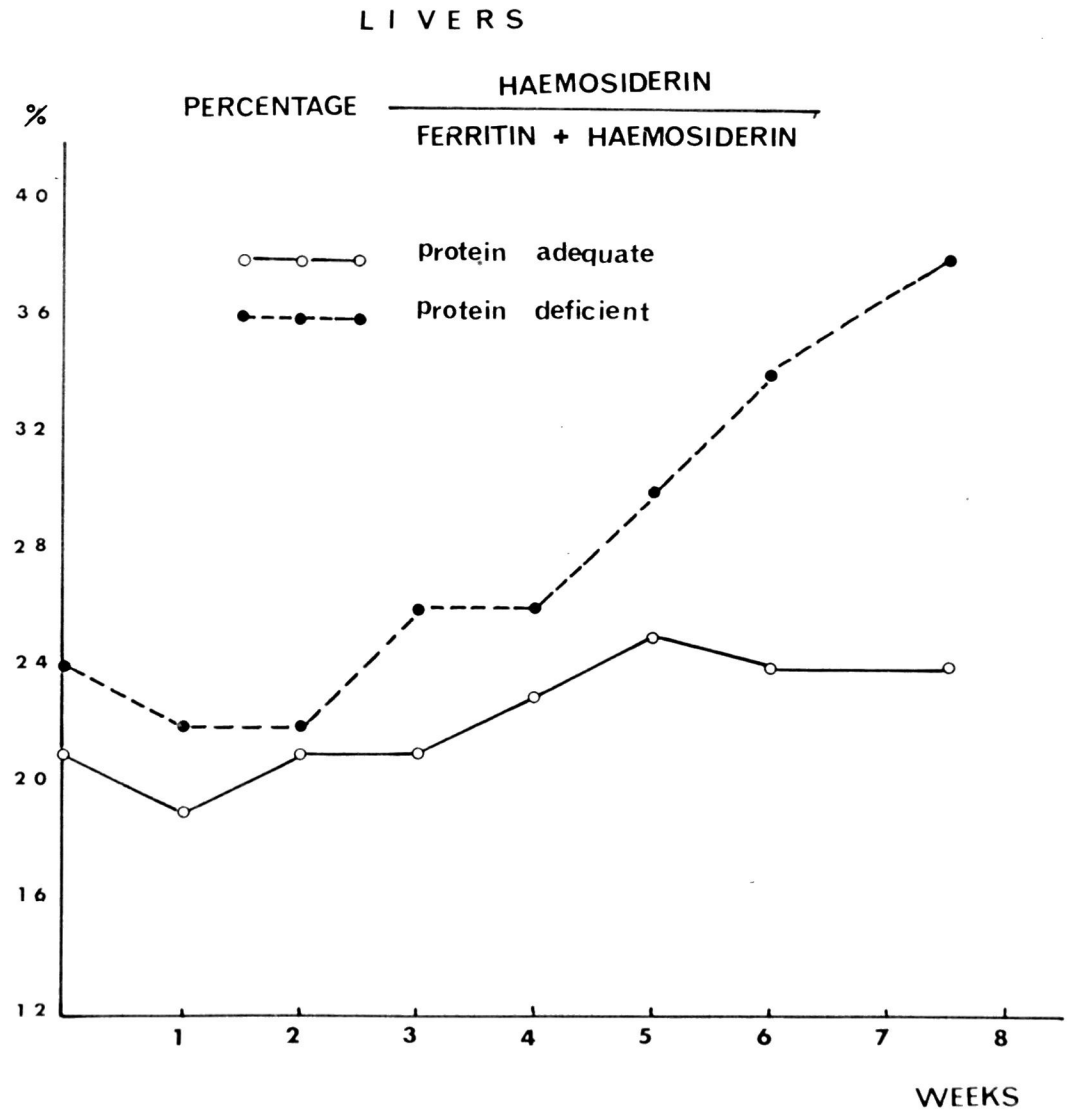


☆ Each Point is an average of 6 rats.

It is demonstrated graphically in Fig. 50 that after seven weeks the total non-haem iron in the livers of protein-deficient rats rose to about 2.6 mg., while in the normal it was about 1.2 mg. The percentage of this non-haem iron stored as haemosiderin in the protein-deficient rats rose gradually after the second week from an initial value of about 24% to reach a value of 38% in the seventh week. In the normal rats it fluctuated between 19 - 25% (Fig. 51). There was a significant difference between the two groups after the fourth week. The average for all the normal rats after the fourth week was 24% (S.E.$\pm$ 0.55), for the protein-deficient it was 33% (S.E.$\pm$ 1.65). The difference of 9% with standard error of $\pm$ 1.7 is obviously very significant.

The initial ratio of haemosiderin to ferritin iron was 1:3. At the end of the experiment it remained at 1:3 in the normal rats and was doubled (2:3) in the protein-deficient.

Fig. **5 1**



☆ Each Point is an average of 6 rats.

It was, thus, evident again that although the excess iron was deposited both as ferritin and haemosiderin in the protein deficient rats, a greater part was deposited as haemosiderin.

It is observed that the initial amounts of storage iron in the spleens and livers of this group of big rats were greater than those of the smaller rats used in the previous experiment. In general, however, this experiment provided close confirmation of the results of the previous one.

EXPERIMENT IV IRON STORAGE AND PROTEIN DEFICIENCY (C)

The Effect of High Iron Intake

The experiments described so far suggest that the protein deficient rat still synthesizes apoferritin, probably in greater quantities than the normal animal. It seemed of interest to study the effect of imposing an even greater strain on the iron storage system by feeding protein-deficient rats (with suitable controls) on a diet rich in iron.

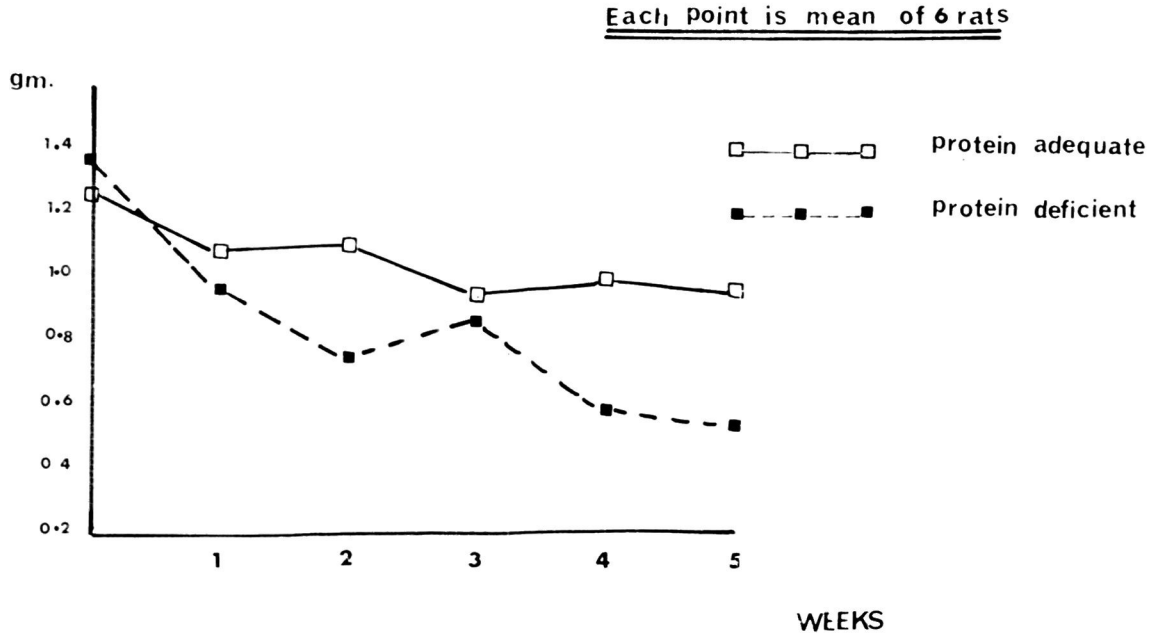
Materials and method

Big rats weighing between 305 and 395 grams (average 340 grams) were divided at random into two groups of 36. Group A was fed on Diet "A" and group B on Diet C (Table 1). To each diet 6% ferric citrate was added replacing an equal amount of water. Each rat received an equivalent of 200 mg. of iron per day, but after three weeks the protein-deficient rats actually ingested about 150 mg. as they consumed less food.

Commencing one day after the beginning of the experiment six rats from each group were killed weekly for analysis.

WEIGHTS OF SPEENS

fig. 5 2



Results and observations

Spleen analysis

Weight

The weights of the spleens declined to about 0.55 grams in the protein-deficient rats after five weeks; the maximum loss, again, occurred in the first two weeks, (Fig. 52). In the rats on protein-adequate diet, the weights varied between 0.95 and 1.36 grams (average 1.05 g. S.D. $\pm$ 0.16) which closely resembles the average in the previous experiments.

Ferritin

The initial ferritin iron concentration in both groups was about 13 mg/100 grams which was the same initial value observed in the previous experiment with rats of the same weight group.

In both groups of rats the ferritin continuously increased, but at a greater rate in the protein-deficient, reaching 61 mg./100 grams in five weeks. In the control rats it was 50 mg./100 grams. The total amount of ferritin in the spleens of the protein-deficient had, thus, increased about three times in five weeks.

fig. 53

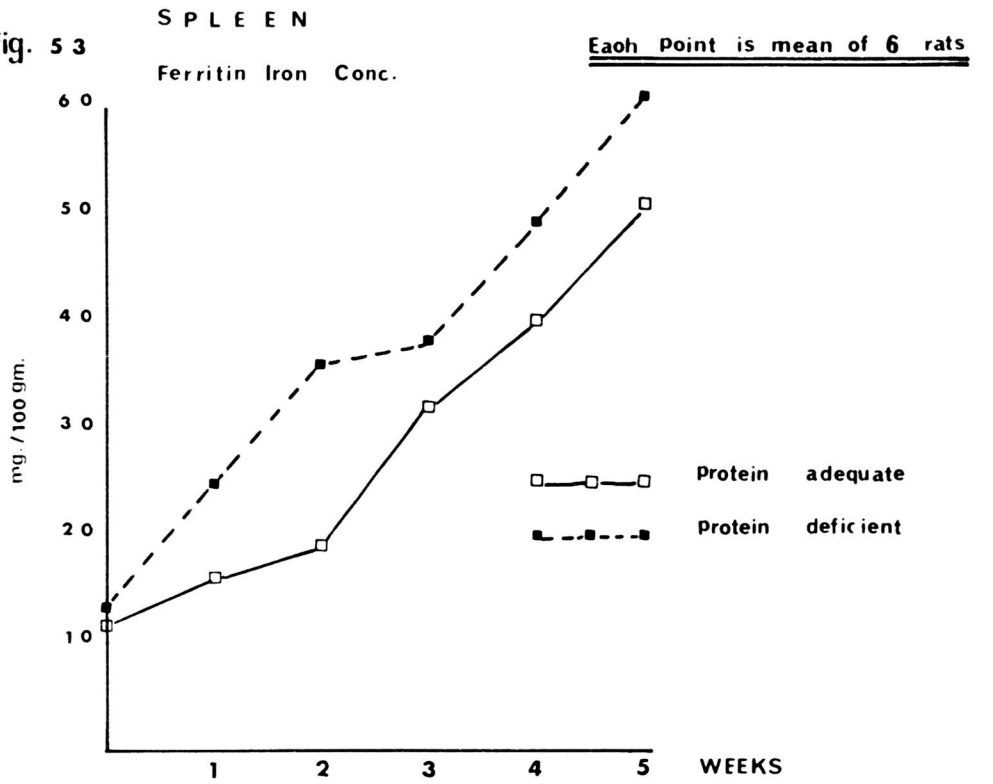
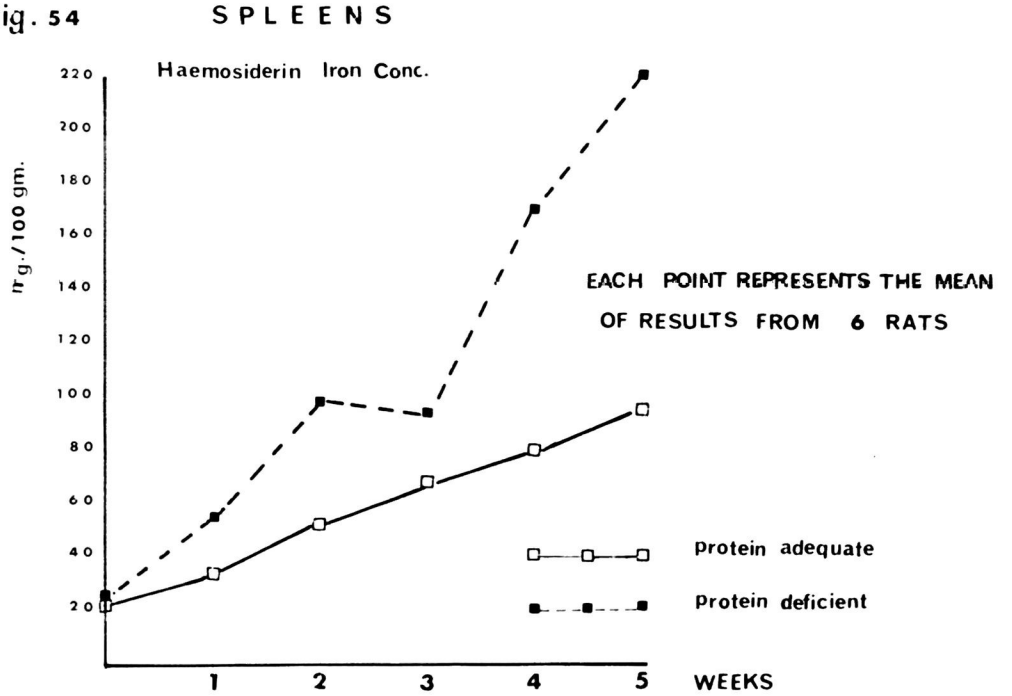

SPLEEN
Ferritin Iron Conc.
Eaoh Point is mean of 6 rats
mg./100 gm.
60
50
40
30
20
10
1
2
3
4
5
WEEKS
Protein adequate
Protein deficient

fig. 54


SPLEENS
Haemosiderin Iron Conc.
mg./100 gm.
220
200
180
160
140
120
100
80
60
40
20
1
2
3
4
5
WEEKS
EACH POINT REPRESENTS THE MEAN
OF RESULTS FROM 6 RATS
protein adequate
Protein deficient

Haemosiderin

There was a more striking increase in the haemosiderin concentration in both groups, more so in the protein-deficient one. Starting at an initial value of 25 mg./100 grams, it increased to 90 in the normal and up to 220 mg./100 grams in the protein-deficient in five weeks (Fig. 54). At the same period in the previous experiment when normal iron was given, the values were 30 and 180 mg./100 grams for the normal and protein-deficient rats respectively.

The total non-haem iron in the spleens (Fig. 55) did not exhibit the striking difference observed in the previous experiment when iron intake was normal. After five weeks it rose to 1.35 mg. in the normal and up to 1.45 mg. in the protein-deficient rats. These values at the same period in the previous experiment amounted to 0.5 and 1.1 mg. in the normal and protein-deficient rats respectively.

fig. 55

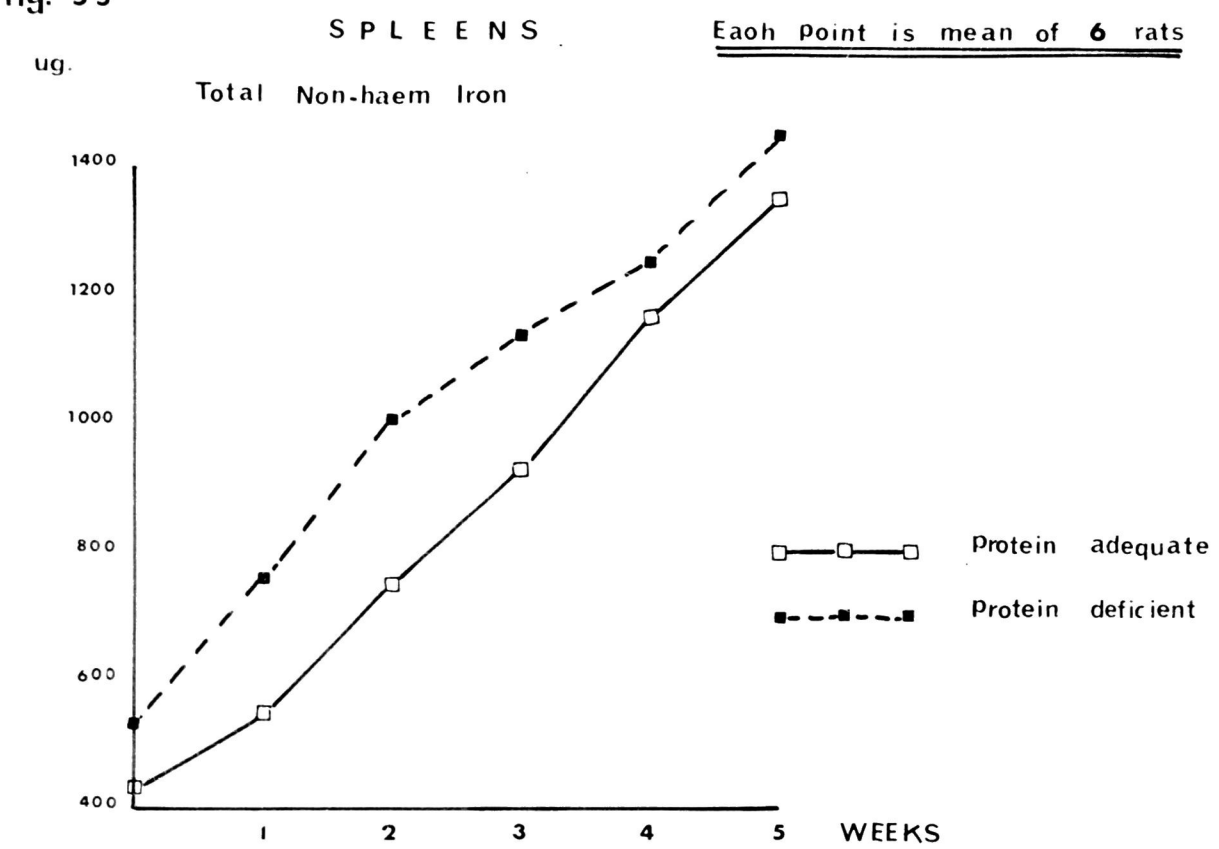
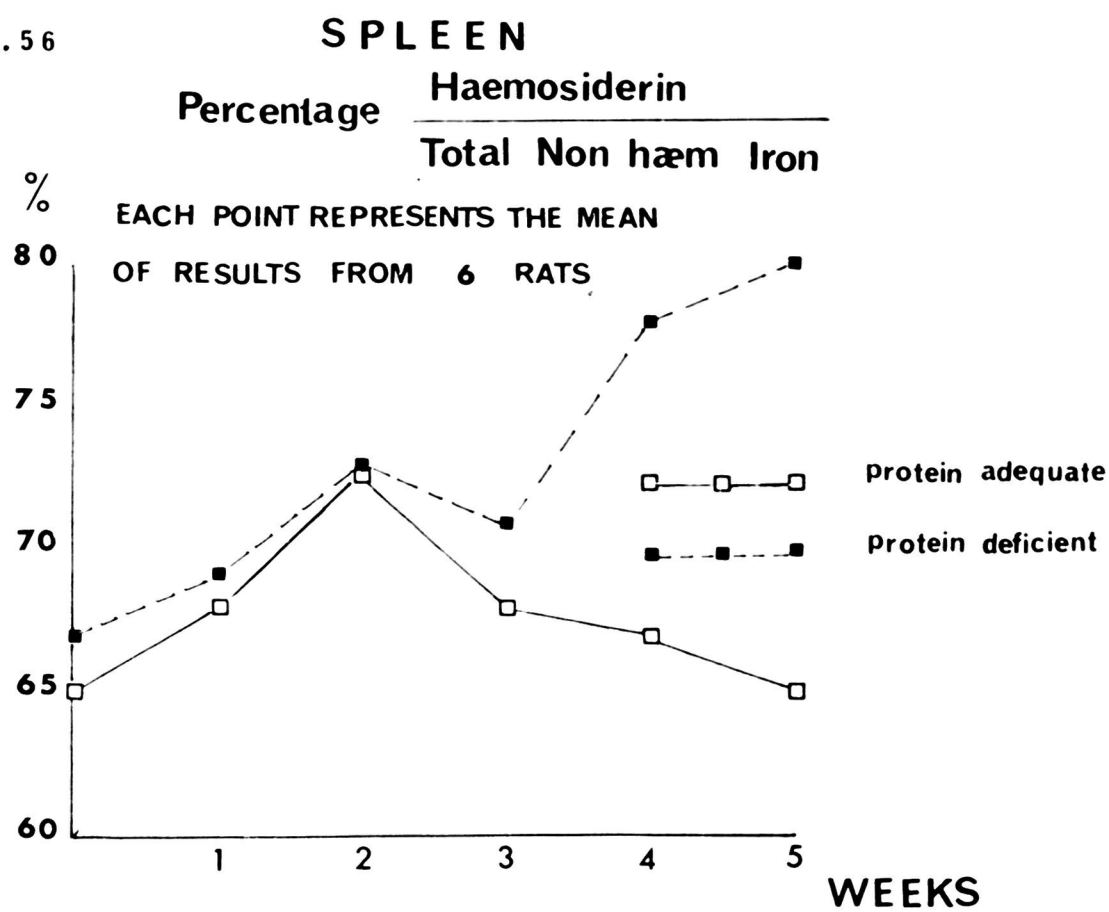

SPLEENS
Eaoh Point is mean of 6 rats
ug.
Total Non-haem Iron
1400
1200
1000
800
600
400
1
2
3
4
5
WEEKS
Protein adequate
Protein deficient

fig. 56


SPLEEN
Percentage Haemosiderin / Total Non hæm Iron
%
EACH POINT REPRESENTS THE MEAN
OF RESULTS FROM 6 RATS
80
75
70
65
60
1
2
3
4
5
WEEKS
protein adequate
protein deficient

The percentage of non-haem iron present as haemosiderin in both groups was initially about 66%. In the normal group it gradually increased to reach a value of 73% in the second week and then gradually decreased to its initial value at five weeks. In the protein-deficient, except for the third week, it progressively increased to reach a value of 80% (Fig. 56). In the first two weeks there was no significant difference between the two groups. After that the average for all the normal rats was 66% S.E. $\pm$ 0.76. For the protein-deficient it was 76% S.E. $\pm$ 0.96. A difference of 10% with standard error of $\pm$ 1.2 is obviously highly significant.

The ratio of haemosiderin to ferritin in the spleen was initially 2:1 in both groups, reaching 9:5 after five weeks in the normal and 9:2½ in the protein-deficient rats.

It was, again, evident that although the protein deficient animal stored excess iron both as ferritin and haemosiderin, it was deposited preferentially as haemosiderin. It was surprising, however, that these rats were capable of maintaining a high level of ferritin in the spleen. In the normal rats the excess iron was stored as ferritin and haemosiderin in the same initial ratio.

fig. 57

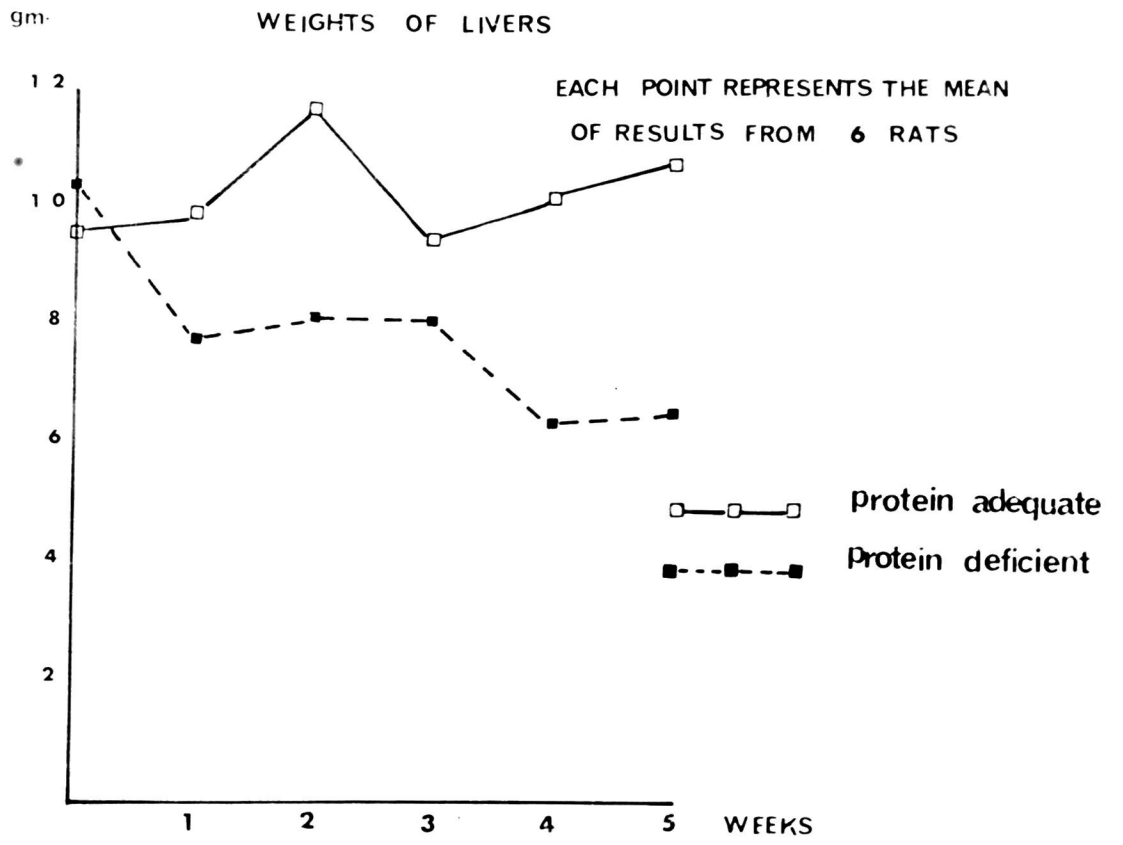

gm.
WEIGHTS OF LIVERS
EACH POINT REPRESENTS THE MEAN
OF RESULTS FROM 6 RATS
12
10
8
6
4
2
1
2
3
4
5
WEEKS
Protein adequate
Protein deficient

fig. 58

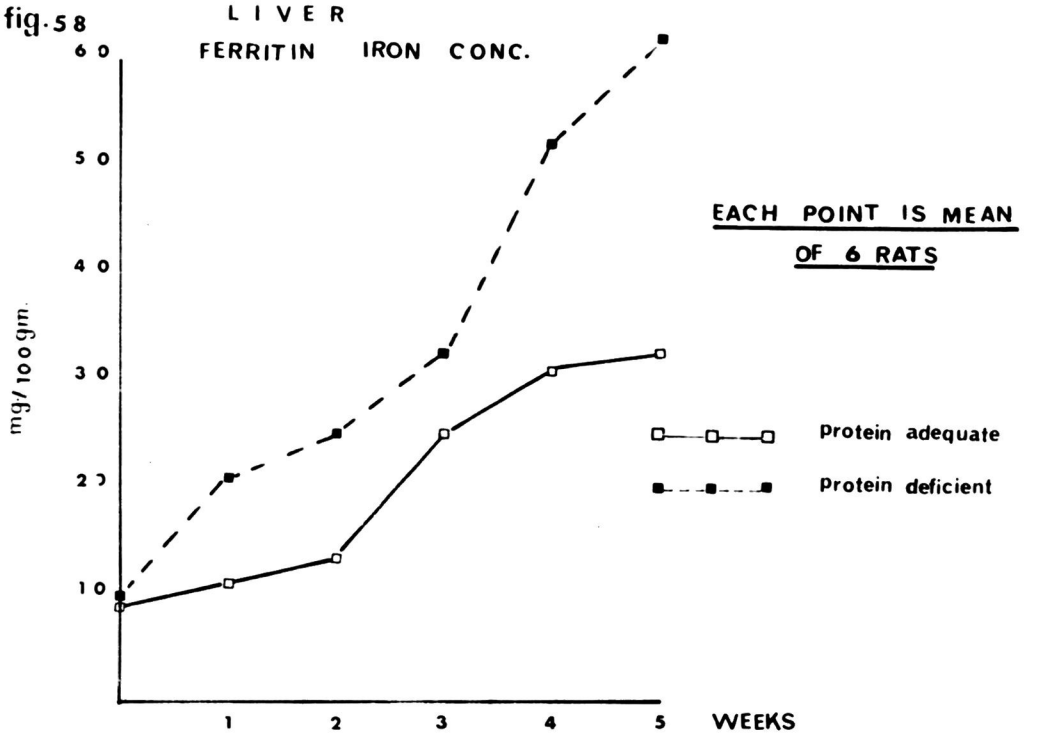
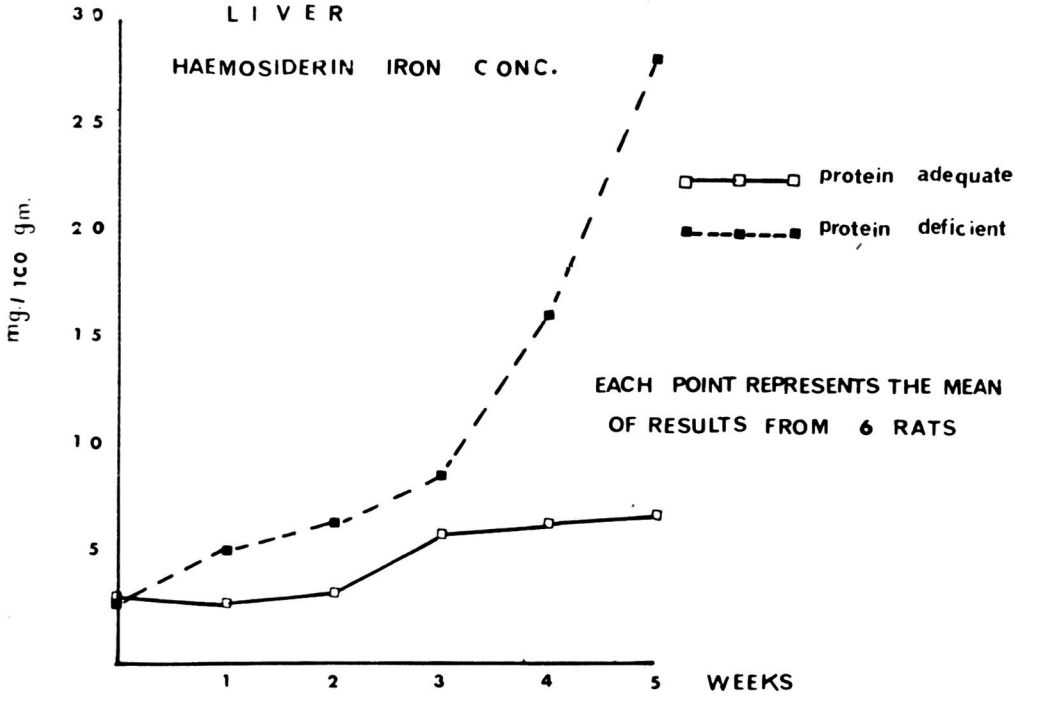

LIVER
FERRITIN IRON CONC.
60
50
40
30
20
10
mg/100gm.
1
2
3
4
5
WEEKS
EACH POINT IS MEAN OF 6 RATS
Protein adequate
Protein deficient

fig. 59


LIVER
HAEMOSIDERIN IRON CONC.
30
25
20
15
10
5
mg./100 gm.
1
2
3
4
5
WEEKS
Protein adequate
Protein deficient
EACH POINT REPRESENTS THE MEAN OF RESULTS FROM 6 RATS

Liver analysis

Weight

As in the previous experiment liver weights of the protein-deficient rats decreased to about 7 grams, the main loss occurring in the first week, with slight loss up to the fourth week (Fig. 57). In the normal group it remained at a constant range of 9.6 - 11.7 grams, average 10.3 grams (S.D.$\pm$ 1.4).

Ferritin

In both groups there was a gradual rise in ferritin iron concentration from an initial level of about 9 mg./100 grams to 32 (three and one half fold increase) in the normal and up to 64 mg./100 grams (seven-fold increase) in the protein-deficient rats in five weeks, (Fig. 58).
It was thus evident that in the liver, too, the total ferritin increased more than four times the initial value in the protein deficient animals, rising from about 0.90 mg. to about 4.22 mg.

Fig. 60

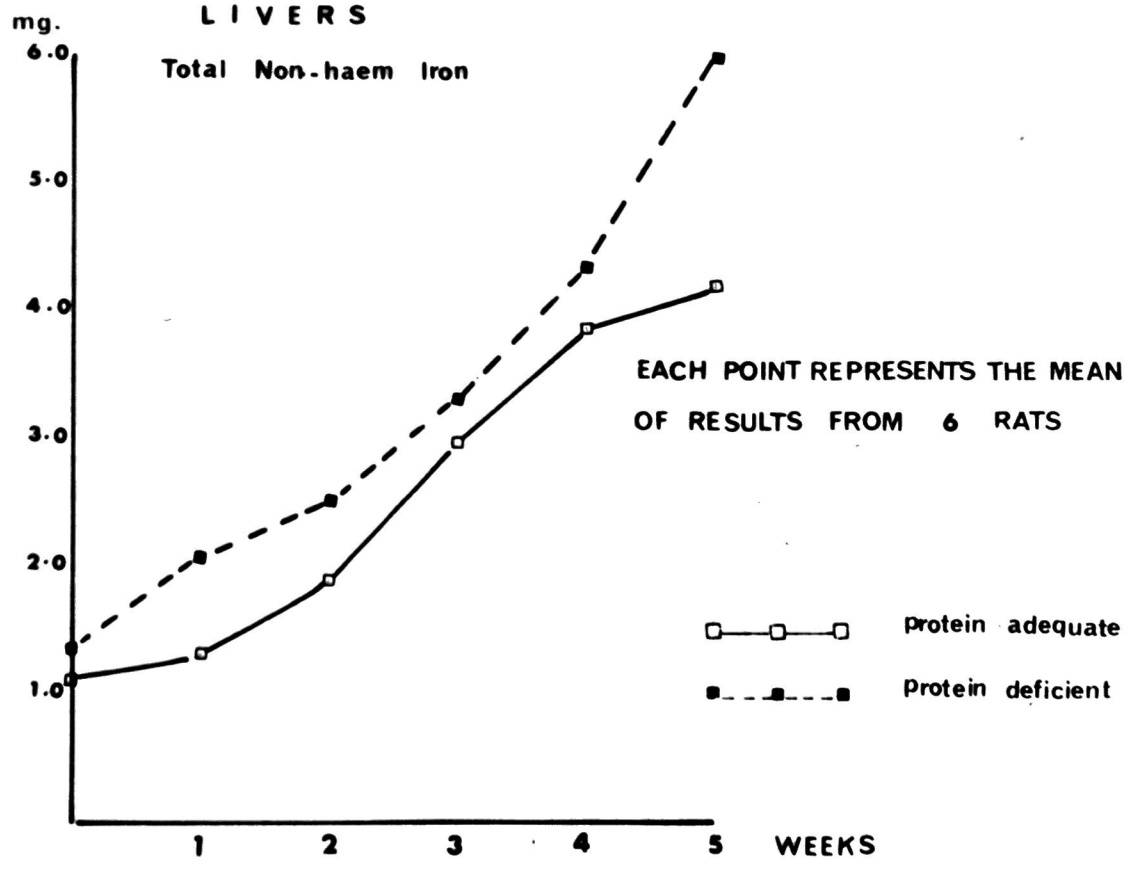
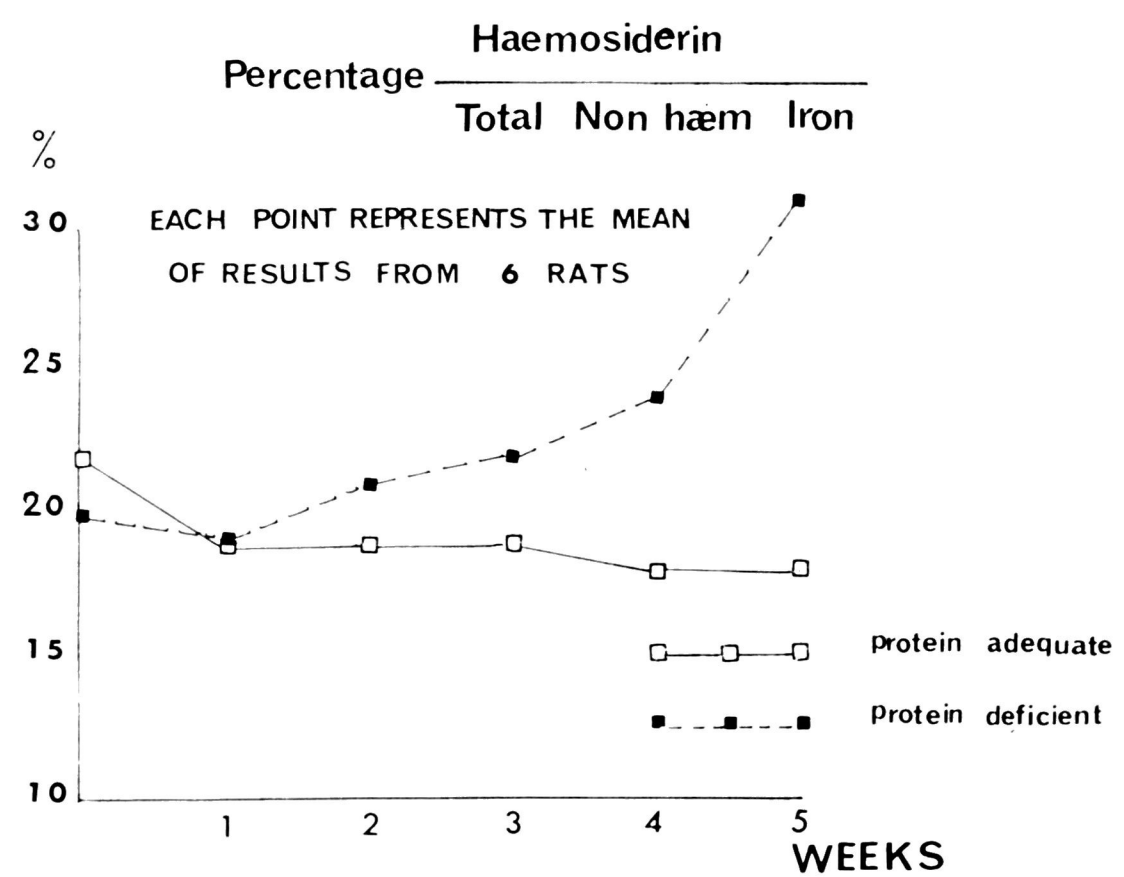

LIVERS
Total Non-haem Iron
mg.
6.0
5.0
4.0
3.0
2.0
1.0
1
2
3
4
5
WEEKS
EACH POINT REPRESENTS THE MEAN
OF RESULTS FROM 6 RATS
Protein adequate
Protein deficient

fig 61


LIVER
Percentage Haemosiderin / Total Non hæm Iron
%
EACH POINT REPRESENTS THE MEAN OF RESULTS FROM 6 RATS
30
25
20
15
10
1
2
3
4
5
WEEKS
Protein adequate
Protein deficient

Haemosiderin

A significant rise in haemosiderin concentration was also observed (Fig. 59), rising from the normal initial level of about 2.6 mg./100 grams to about 7 in the normal and up to about 28 mg./100 grams in the protein-deficient in five weeks.

After the second week there was a significant difference between the two groups as regards the percentage of the storage iron present as haemosiderin (Fig. 61). The average for all the normal rats after the second week was 18% S.E. ± 0.76, and for the protein-deficient it was 26% S.E. ± 1.2. The difference was 8% with standard error of ± 1.9. A similar change in the ratio of haemosiderin to ferritin was observed. It decreased from 0.29 initially to 0.22 in the normal rats while in the protein-deficient it increased to 0.45. The total non-haem iron in the livers of both groups went up when excess iron was added to the diets, reaching a value of 4.2 mg. in the normal and 6.0 mg. in the protein-deficient (Fig. 60).

In agreement with the observations of Hegsted et al. (1949) the normal rats stored more iron in the liver and spleen when given excess iron in the diet than when given normal iron. Although the protein deficient rats still appeared to absorb more* than the normal, the difference was less striking than on the normal iron diet. As before most of the excess iron in the spleen, and some of that in the liver was stored as haemosiderin. In both organs, however, ferritin formation was well maintained.

*Footnote: as far as can be judged from the analysis of the storage organs.

These experiments on iron storage in the liver and spleen confirm that the protein-deficient rat deposits much more iron in the storage organs. They also show that a substantial proportion of the additional iron is found as ferritin, even when iron uptake is greatly increased by the inclusion of excessive amounts of iron in the diet.

DISCUSSION

Investigations of the relationships of protein-deficiency and iron metabolism are not without relevance to practical clinical medicine and public health problems, since protein deficiency and siderosis are known to co-exist in man (Strachan, 1929; Gillman et al. 1945; Higginson et al. 1953; Gillman et al. 1957). Whether the excess iron is stored in the normal protein-containing complexes, in spite of the impossibility of maintaining the normal levels of most proteins, is an extremely fascinating question.

I. IRON ABSORPTION

The results outlined in the experimental section confirm conclusively the previous observations (Kaufman, Klavis, and Kinney, 1958; Gillman et al. 1958; Rather, 1956; Gillman et al. 1959; Wack and Wyatt, 1959; Wöhler and Zell, 1960) that protein-deficiency markedly increases the capacity to absorb iron and leads to siderosis. Until the problem of the regulation of iron absorption is clearly resolved, the attempts to explain the factors involved in the regulation of absorption under abnormal conditions, such as protein-deficiency, anaemias with high iron stores, haemochromatosis etc., are only speculative, but a reasonable assumption is that the postulated mucosal barrier of Hahn et al. (1943) does not exist under these conditions. Hegsted et al. (1949) explained the increased absorption on the basis of low phosphorus content of their protein-deficient diet.

The results of the present experiments show that there are at least other factors involved. When iron was administered to fasting animals in the form of iron-ascorbic acid solution, the protein-deficient rats still absorbed more iron than the normal. Moreover rats fed on casein diet - phosphoprotein - and rats on laboratory stock diet behaved similarly. There seems to be a direct relation between the degree of protein-deficiency and iron absorption. The rats absorbed progressively more iron as they became more protein-deficient, at least, within the limits of the duration of the present experiments. This was observed from the radioiron absorption experiments and from the analysis of the storage iron in the tissues.

Wack and Wyatt (1959) determined the areas of radio-iron absorption in the gastro-intestinal tract of the rat under different dietary states. They found an extension of the anatomical zone of intestinal iron absorption in animals on low-protein, high-iron diet and in iron deficiency states.

Gillman et al. (1951) Beutler (1957) Beutler et al. (1958) attributed the increased absorption in protein-deficiency to its effect on intracellular metabolism and intracellular enzymes, but they did not produce any direct evidence to support their views.

There is considerable evidence from observations in man and experimental animals on the relation of the exocrine pancreas to iron absorption and siderosis. Ligation of the pancreatic ducts and resulting pancreatic degeneration in rats, dogs and cats led to increased iron absorption and siderosis (Taylor, Stiven and Reid, 1935; Gillman, Gillman, Mandelstam and Gilbert, 1947;

Kinney, Finch, Kaufman, Hegsted and Partington, 1950). Moreover pancreatic degeneration due to pyridoxine deficiency in rats also led to increased iron absorption and siderosis of the liver (Cartwright et al. 1944). Davis and Badenock (1962) found that patients with chronic pancreatitis and pancreatic fibrosis showed greatly enhanced iron absorption which decreased when pancreatin was given. Smith (1964) reported similar observations in children with cystic fibrosis of the pancreas.

Degeneration and fibrosis of the pancreas in man due to protein-deficiency were reported by several workers, in Kwashiorkor(Davies, 1948; Zuidema, 1959; and Sharper, 1960) and as a result of war-time difficulties (Veghelyi, 1948; Veghelyi et al. 1950). Similar effects were observed in protein-deficient animals (Grossman, Greengard and Ivy, 1943; Friedman and Friedman 1946; Kristal, 1947; Veghelyi et al. 1950; Miller and Rigdon, 1952; Wachstein and Meisel, 1954). Wachstein and Meisel (1954) also

found that raising the casein content of the diet above 4% prevented pancreatic damage. Moreover, Kristal (1947) found siderosis of the livers of the protein-deficient rats with pancreatic degeneration. This pancreatic lesion due to protein-deficiency may well be one of the factors involved in the increased absorption of iron and siderosis, in experimental animals and perhaps in the South African Bantu as well, since Kwashiorkor is widespread among the children in these regions.

Histological sections of the pancreas from protein-deficient rats used in these experiments showed scattered small foci in which acini seemed to have atrophied and in some cases broken up, their cellslying singly or in small groups. These cells were often shrunken and their cytoplasm might be vacuolated. There was no marked inflamatory cell infiltration and polymorphs were not a feature of the condition. There was no fibrosis and the islets of Langerhans appeared normal. These findings are essentially similar to those reported in the literature.

Light Microscope Pictures of the Small Intestine

Fig. 62 Normal x 80

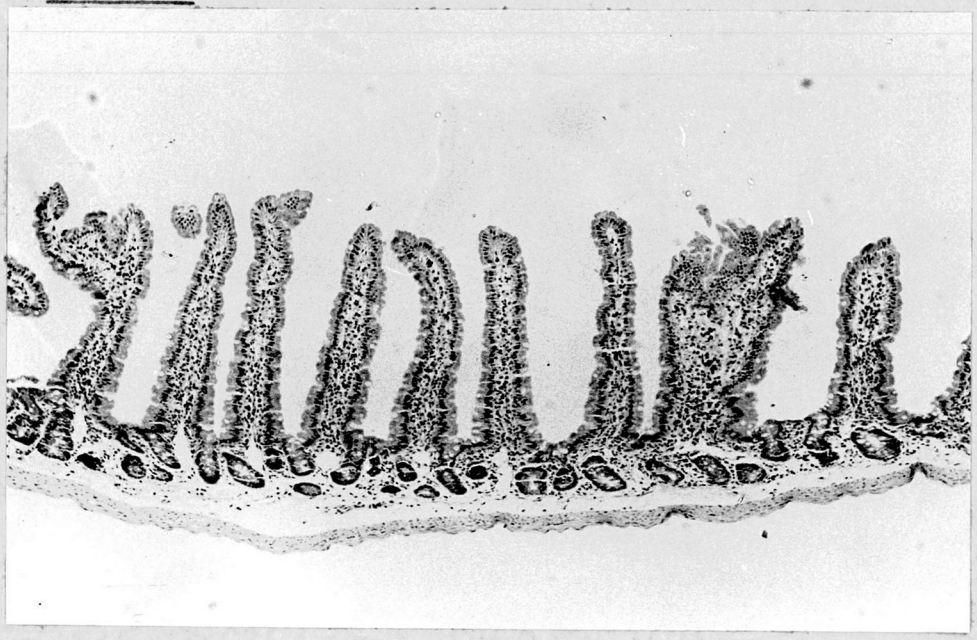
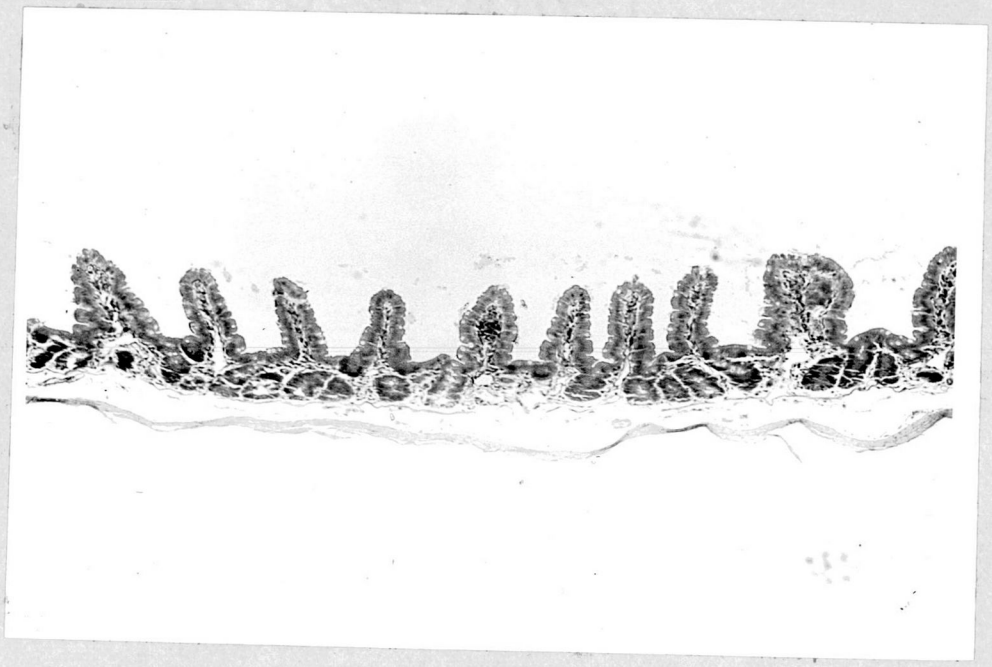


Fig. 63 Protein-Deficient x 80



The suggestion of Charlton et al. (1963) that iron incorporated into ferritin in the intestinal mucosal cells is lost from the body when the cells exfoliate is of great interest. Again Ramalingaswami (1964) has shown in monkeys that the intestinal cell turnover is reduced to less than half in protein deficiency, and the villi become short and stunted. This decrease in the intestinal cell turnover may allow for more ferritin iron to be released into the circulation before it is finally lost by exfoliation. Whether the slight anaemia caused by protein-deficiency and possible hypoxia play a part in the increased absorption is difficult to say.

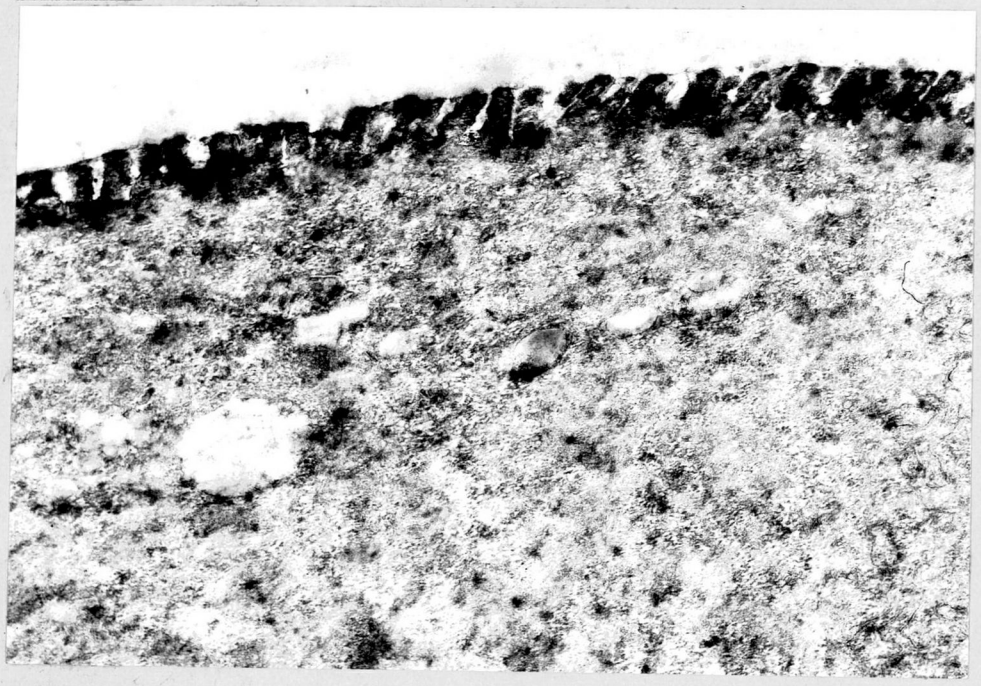
The histological appearance* of the intestine under the light microscope (Figs. 62, 63) was very striking

*Footnote: Histological examinations of the tissues were kindly undertaken by Dr. M. MacDonald, and Electron microscopy of the intestine by Dr. B.K. Patnaik both from the Pathology Department, University of Edinburgh.

Electron Microscope Pictures of the Small Intestine

Fig. 64 Normal x 3,000

Fig. 65 Deficient x 32,000



The villi were markedly shorter than normal and spaced further apart. Electron microscopy showed that the microvilli were also short and stunted (Figs. 64, 65). Ramalingaswarmi (1964) reported similar results in monkeys.

The livers of protein-deficient rats fed excess iron showed demonstrable iron mainly in the Kupffer cells with smaller amounts in the parenchymal cells. The livers were fatty, with increased connective tissue in the portal tracts but no evidence of fibrosis or cirrhosis. The architecture of the liver lobules was otherwise unaltered.

II. IRON STORAGE

In these experiments the protein-deficient animals lost a great deal of weight and showed the expected decline in haemoglobin, transferrin, and in the weights of the visceral organs. The synthesis of a great many proteins was undoubtedly impaired.

Surprisingly, it appears that apoferritin was an exception to this rule. Although the excess iron absorbed was deposited mainly as haemosiderin iron in the spleen, the protein-deficient rats were able to build up a high concentration of ferritin iron in both liver and spleen, even more so when excess iron was included in the diet. In some cases there was a more than four-fold increase in the amount of ferritin iron, and the change in concentration was even greater. This alone does not necessarily imply increasedsynthesis of apoferritin, for Mazur, Litt and Shorr (1950) reported that various grades of ferritin with iron contents ranging from very low values to 23% could be obtained by centrifugal techniques, and as long ago as 1944, Rothen showed the presence of some free apoferritin in ferritin preparations. It seems very likely

that some of the increase in ferritin iron observed in protein-deficient rats might be accounted for by more efficient use of apoferritin.

On the other hand it is known that apoferritin synthesis is stimulated by the presence of iron and that when iron is incorporated into ferritin, much of it is found with newly synthesized apoferritin (Fineberg and Greenberg, 1955). The increase in ferritin iron observed in the protein-deficient rats is so large, that it seems highly probably that apoferritin synthesis must be at least maintained and perhaps even increased. The experimental investigation of this point would be a major task, but clearly an important one, because the implication is that apoferritin is a protein which may require to be preferentially synthesized in protein-deficiency.

ACKNOWLEDGEMENTS

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